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(54) **Hydroxyalkyl starch derivatives and process for their preparation**

(57)   The invention relates to a method for the preparation of a hydroxyalkyl starch derivative which comprises reacting hydroxyalkyl starch (HAS) via the optionally oxidised reducing end of the HAS with the amino group M of a crosslinking compound which, apart from the amino group, comprises a specifically protected carbonyl group, namely an acetal group or a ketal group.

EP 2 070 950 A1

Description

[0001] The invention relates to a method for the preparation of a hydroxyalkyl starch derivative which comprises reacting hydroxyalkyl starch (HAS) via the optionally oxidised reducing end of the HAS with the amino group M of a crosslinking compound which , apart from the amino group, comprises a specifically protected carbonyl group, namely an acetal group or a ketal group. The method may further comprise a reaction of the HAS derivative thus obtained with the amino group of a biologically active compound via alkylation, preferably via reductive amination. Moreover, the invention relates to the HAS derivatives obtainable or obtained by the inventive process and to specific HAS derivatives as such. The invention also relates to pharmaceutical compositions comprising the HAS derivatives containing the biologically active compound, these HAS derivates therapeutic or prophylactic agent and the use of specific HAS derivatives for the preparation of medicaments.

[0002] Hydroxyalkyl starch (HAS), in particular hydroxyethyl starch (HES), is a substituted derivative of naturally occurring carbohydrate polymer amylopectin, which is present in corn starch at a concentration of up to 95 % by weight, and is degraded by alpha-amylase in the body. HES, in particular, exhibits advantageous biological properties and is used as a blood volume replacement agent and in hemodilution therapy in the clinics (Sommermeyer et al., 1987, Krankenhauspharmazie, 8(8), 271-278; Weidler et al., 1991, Arzneimittelforschung/Drug Res., 41, 494-498).

[0003] Some ways of producing a hydroxyethyl starch derivative are described in the art.

[0004] DE 26 16 086 discloses the conjugation of hemoglobin to hydroxyethyl starch wherein, in a first step, a crosslinking agent, e.g. bromocyane, is bound to hydroxyethyl starch and subsequently hemoglobin is linked to the intermediate product.

[0005] One important field in which HES is used is the stabilization of polypeptides which are applied, e.g., to the circulatory system in order to obtain a particular physiological effect. One specific example of these polypeptides is erythropoietin, an acid glycoprotein of approximately 34,000 kDa which is essential in regulating the level of red blood cells in the circulation.

[0006] A well-known problem with the application of polypeptides and enzymes is that these proteins often exhibit an unsatisfactory stability. Especially erythropoietin has a relatively short plasma half live (Spivak and Hogans, 1989, Blood 73, 90; McMahon et al., 1990, Blood 76, 1718). This means that therapeutic plasma levels are rapidly lost and repeated intravenous administrations must be carried out. Furthermore, in certain circumstances an immune response against the peptides is observed.

[0007] It is generally accepted that the stability of polypeptides can be improved and the immune response against these polypeptides is reduced when the polypeptides are coupled to polymeric molecules.

[0008] WO 94/28024 discloses that physiologically active polypeptides modified with polyethylene glycol (PEG) exhibit reduced immunogenicity and antigenicity and circulate in the bloodstream considerably longer than unconjugated proteins, i.e. have a longer clearance rate. However, PEG-drug conjugates exhibit several disadvantages, e.g. they do not exhibit a natural structure which can be recognized by elements of in vivo degradation pathways. Therefore, apart from PEG-conjugates, other conjugates and protein polymerates have been produced.

[0009] WO 02/080979 discloses compounds comprising a conjugate of an active agent and a hydroxyalkyl starch wherein active agent and hydroxyalkyl starch are either linked directly or via a linker compound. As far as the direct linkage is concerned, the reaction of active agent and hydroxyalkyl starch is carried out in an aqueous medium which comprises at least 10 wt.-% of water. No examples are given which are directed to a hydroxyalkyl starch linked to crosslinking compound via an amino group of said crosslinking compound wherein the crosslinking compound further contains a protected carbonyl group. Additionally, no examples are given showing a HAS derivative which is obtained by reacting said HAS derivative via said carbonyl group with an amino group of an biologically active agent.

[0010] WO 03/074087 discloses hydroxyalkyl starch protein conjugates in which the bonding between a hydroxyalkyl starch molecule and a protein is covalent and is the result of a coupling of a terminal aldehyde group of the hydroxyalkyl starch or a functional group which resulted from the reaction of said aldehyde group with a functional group of a protein.

[0011] WO 03/074088 discloses hydroxyalkyl starch conjugates with a low molecular weight compound in which the bonding between the hydroxyalkyl starch and the low molecular weight compound is covalent and is the result of a coupling of a terminal aldehyde group of the hydroxyalkyl starch or a functional group which resulted from the reaction of said aldehyde group with a functional group of a protein.

[0012] WO 2005/014024 discloses polymers functionalized by an aminooxy group or a derivative thereof, conjugates, wherein the functionalized polymers are covalently coupled with a protein by an oxime linking group, a process for preparing the functionalized polymers, a process for preparing the conjugates, functionalized polymers as obtainable by the process of the present invention, conjugates as obtainable by the process, and pharmaceutical compositions comprising at least one conjugate and the use of said conjugates and compositions for the prophylaxis or therapy of the human or animal body.

[0013] WO 2005/092390 discloses conjugates of hydroxyalkyl starch and a protein wherein these conjugates are formed by a covalent linkage between the hydroxyalkyl starch or a derivative of the hydroxyalkyl starch and the protein

and a method of producing these conjugates and the use of these conjugates.

[0014] WO 2004/024777 discloses hydroxyalkyl starch derivates, particularly hydroxyalkyl starch derivatives obtainable by a process in which hydroxyalkyl starch is reacted with a primary or secondary amino group of a linker compound. According to an especially preferred embodiment, WO 2004/024777 discloses hydroxyalkyl starch derivatives obtainable by a process according to which hydroxyalkyl starch is reacted with a primary or secondary amino group of a linker compound and the resulting reaction product is reacted with a polypeptide, preferably with a glycoprotein and especially preferably with erythropoietin, via at least one other reactive group of the linker compound. A hydroxyalkyl starch which is especially preferred is hydroxyethyl starch. According to WO 2004/024777, the hydroxyalkyl starch and preferably the hydroxyl ethyl starch is reacted with the linker compound at its reducing end which is not oxidised prior to the reaction.

[0015] WO 2004/024776 discloses hydroxyalkyl starch derivates, particularly hydroxyalkyl starch derivatives obtainable by a process in which hydroxyalkyl starch is reacted with a primary or secondary amino group of a crosslinking compound or with two crosslinking compounds wherein the resulting hydroxyalkyl starch derivative has at least one functional group X which is capable of being reacted with a functional group Y of a further compound and wherein this group Y of the further compound is an aldehyde group, a keto group, a hemiacetal group, an acetal group, or a thio group. According to an especially preferred embodiment, WO 2004/024776 relates to hydroxyalkyl starch derivatives obtainable by a process according to which hydroxyalkyl starch is reacted with a primary or secondary amino group of a crosslinking compound, the resulting reaction product optionally being further reacted with a second crosslinking compound, wherein the resulting hydroxyalkyl starch derivative has at least one functional group X which is capable of being reacted with a functional group Y of a further compound and wherein this group Y is an aldehyde group, a keto group, a hemiacetal group, an acetal group, or a thio group, and the resulting reaction product is reacted with a polypeptide, preferably with a polypeptide such as AT III, IFN-beta or erythropoietin and especially preferably with erythropoietin, which comprises at least one of these functional groups Y. A hydroxyalkyl starch which is especially preferred is hydroxyethyl starch. According to WO 2004/024776 the hydroxyalkyl starch and preferably the hydroxyethyl starch is reacted with the linker compound at its reducing end which is optionally oxidised prior to the reaction.

[0016] WO 2005/092928 discloses conjugates of hydroxyalkyl starch, preferably hydroxyethyl starch, and a protein, wherein these conjugates are formed by a reductive amination reaction between at least one aldehyde group of the hydroxyalkyl starch or of a derivative of the hydroxyalkyl starch, and at least one amino group of the protein, so that the hydroxyalkyl starch or the derivative thereof is covalently linked to the protein via an azomethine linkage or a aminomethylene linkage. WO 2005/092928 also relates to a method of producing these conjugates and specific uses of the conjugates.

[0017] US 2006/0194940 A1 discloses water-soluble polymer alkanals. Among others, protected aldehyde reagents disclosed which are reacted with a polymer. While poly(saccharides) are generically mentioned, especially preferred polymers are polyethylene glycols. Starches or, in particular, modified starches such as hydroxyalkyl starches are not disclosed in US 2006/0194940 A1. Consequently, US 2006/0194940 A1 contains no disclosures concerning specific ways of coupling a given linker compound to hydroxyalkyl starch. The same applies to US 7,157,546 B2, EP 1 591 467 A1 and WO 2004/022630 A2.

[0018] US 6,916,962 B2 discloses an aminoacetal crosslinking compound in unprotected and protected form. No disclosure is contained in this document relating to a possible coupling of this crosslinking compound with polymers other than polyethylene glycols. In particular, starches, let alone modified starches such as hydroxyalkyl starches are not disclosed in US 6,916,962 B2. Consequently, US 6,916,962 B2 contains no disclosures concerning specific ways of coupling a given linker compound to hydroxyalkyl starch. The same applies to US 6,956,135 B2 and WO 03/049699 A2.

[0019] US 5,990,237 discloses structures containing a protected aldehyde group. Compounds comprising these structures are preferably coupled to polyethylene glycol, and coupling is carried out via a halide as functional group comprised in the protected aldehyde group containing compounds, which halide group reacts with a hydroxy group of the polyethylene glycol.

[0020] It is an object of the present invention to provide a novel method to obtain hydroxyalkyl starch derivatives.

[0021] It is a further object of the present invention to provide novel HAS derivatives such as HAS derivatives obtained or obtainable by reacting HAS with specifically functionalized crosslinking compounds.

[0022] It is yet another object of the present invention to provide further novel HAS derivatives such as HAS derivatives obtained or obtainable by reacting the HAS derivatives - obtained or obtainable by reacting HAS with specifically functionalized crosslinking compounds - with a suitable functional group of biologically active compound.

[0023] Surprisingly, it was found that it is possible to use, for the preparation of specific HAS derivatives, a crosslinking compound which, on the one hand, can selectively be coupled to the optionally oxidized reducing end of a hydroxyalkyl starch via an amino group and, on the other hand, has - as a second functional group - a fully protected carbonyl group, namely an acetal group or a ketal group. Compared to embodiments where a crosslinking compound is employed having a free aldehyde or keto group or, e.g., a hemiacetal group as functional group, employing such a fully protected group drastically minimises the risk that, during reaction of HAS with the crosslinking compound, undesired oligomerisation or polymerisation between the crosslinking compound molecules takes places. Unexpectedly, it was found that deprotection

of the acetal or ketal group comprised in the resulting HAS derivative is possible without at least partially destroying the specific chemical structure of the hydroxyalkyl starch, in particular the hydroxyethyl starch, being characterised by numerous functional groups such as acetal groups and ether groups. Therefore, the present invention allows for an extremely effective method of preparing a first HAS derivative by minimising the risk of oliogomerisation or polymerisation between the individual crosslinking compound molecules, combined with the possibility of deprotecting the functional groups of the resulting HAS derivatives without at least partial destruction of the HAS structure, in order to provide a HAS derivative allowing for an effective coupling with a biologically active compound.

[0024]   Thus, the present invention relates to a method for the preparation of a hydroxyalkyl starch derivative, comprising

(i) reacting hydroxyalkyl starch (HAS) of formula (I)

(I)
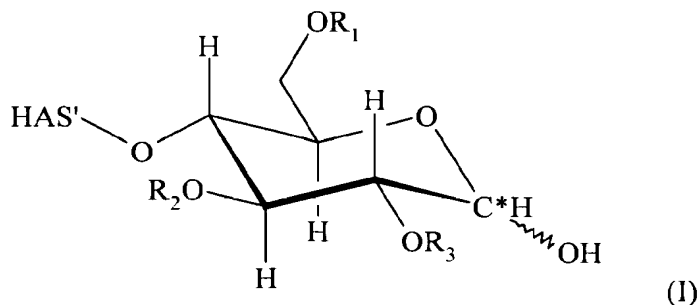
via carbon atom C^* of the reducing end of the HAS with the amino group M of a crosslinking compound according to formula (II)

M-L-A

wherein A is an acetal group or a ketal group; and L is a spacer bridging M and A, wherein C^* is optionally oxidised prior to the reaction of HAS with M,
obtaining a HAS derivative according to formula (III)

(III)
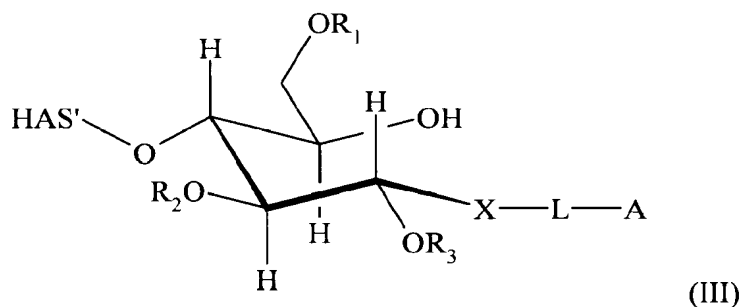
wherein X is the functional group resulting from the reaction of the amino group M with the HAS via carbon atom C^* of the optionally oxidised reducing end of the HAS, and
wherein HAS' is the remainder of the hydroxyalkyl starch molecule, and R_1, R_2 and R_3 are independently hydrogen or a linear or branched hydroxyalkyl group.

[0025]   Further, the present invention relates to a hydroxyalkyl starch (HAS) derivative obtainable or obtained by this method.
[0026]   Moreover, the present invention relates to a hydroxyalkyl starch (HAS) derivative of formula (III)

(III)

wherein A is an acetal or ketal group; L is a spacer bridging X and A;

wherein X is the functional group resulting from the reaction of an amino group M of a crosslinking compound of formula (II)
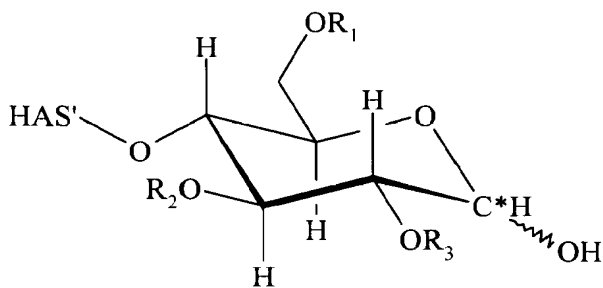
M-L-A

with hydroxyalkyl starch (HAS) of formula (I)

(I)

via carbon atom C^* of the HAS, wherein C^* is optionally oxidised prior to the reaction of HAS with M,

wherein HAS' is the remainder of the hydroxyalkyl starch molecule and R_1, R_2 and R_3 are independently hydrogen or a linear or branched hydroxyalkyl group.

<u>Hydroxyalkyl starch</u>

[0027] In the context of the present invention, the term "hydroxyalkyl starch" (HAS) refers to a starch derivative which has been substituted by at least one hydroxyalkyl group. A preferred hydroxyalkyl starch of the present invention has a constitution according to formula (I')
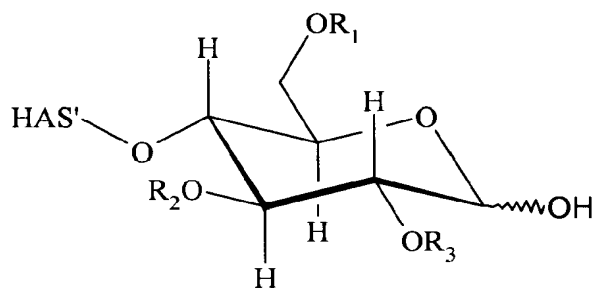
(I')

wherein HAS' is the remainder of the hydroxyalkyl starch molecule and R_1, R_2 and R_3 are independently hydrogen, a linear or branched hydroxyalkyl group or the group

$$-[(CR^1R^2)_mO]_n[CR^3R^4]_o-OH$$
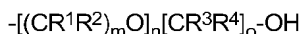
wherein R^1, R^2, R^3, and R^4 are independently selected from the group consisting of hydrogen, and alkyl group, preferably hydrogen and methyl group,

    m is 2 to 4, wherein the residues R^1 and R^2 may be the same or different in the m groups CR^1R^2;
    n is 0 to 20, preferably 0 to 4;
    o is 2 to 20, preferably 2 to 4, wherein the residues R^3 and R^4 may be the same or different in the o groups CR^3R^4.

[0028]   Preferably, R_1, R_2 and R_3 are independently a group -$(CH_2CH_2O)_n$-H, wherein n is an integer, preferably 0, 1, 2, 3, 4, 5, or 6, and in particular, R_1, R_2 and R_3 are independently hydrogen or 2-hydroxyethyl.

[0029]   In formula (I) and (I') the reducing end of the starch molecule is shown in the non-oxidised form and the terminal saccharide unit of HAS is shown in the hemiacetal form which, depending on e.g. the solvent, may be in equilibrium with the (free) aldehyde form. The abbreviation HAS' as used in the context of the present invention refers to the HAS molecule without the terminal saccharide unit at the reducing end of the HAS molecule. This is meant by the term "remainder of the hydroxyalkyl starch molecule" as used in the context of the present invention.

[0030]   The term "hydroxyalkyl starch" as used in the present invention is not limited to compounds where the terminal carbohydrate moiety comprises hydroxyalkyl groups R_1, R_2 and/or R_3 as depicted, for the sake of brevity, in formulas (I) and (I'), but also refers to compounds in which at least one hydroxy group which is present anywhere, either in the terminal carbohydrate moiety and/or in the remainder of the hydroxyalkyl starch molecule, HAS', is substituted by a hydroxyalkyl group R_1, R_2 and/or R_3.

[0031]   Hydroxyalkyl starch comprising two or more different hydroxyalkyl groups is also possible.

[0032]   The at least one hydroxyalkyl group comprised in HAS may contain one or more, in particular two or more hydroxy groups. According to a preferred embodiment, the at least one hydroxyalkyl group comprised in HAS contains one hydroxy group.

[0033]   The expression "hydroxyalkyl starch" also includes derivatives wherein the alkyl group is mono- or polysubstituted. In this context, it is preferred that the alkyl group is substituted with a halogen, especially fluorine, or with an aryl group. Furthermore, the hydroxy group of a hydroxyalkyl group may be esterified or etherified.

[0034]   Furthermore, instead of alkyl, also linear or branched substituted or unsubstituted alkenyl groups may be used.

[0035]   Hydroxyalkyl starch is an ether derivative of starch. Besides of said ether derivatives, also other starch derivatives can be used in the context of the present invention. For example, derivatives are useful which comprise esterified hydroxy groups. These derivatives may be e.g. derivatives of unsubstituted mono- or dicarboxylic acids with 2-12 carbon atoms or of substituted derivatives thereof. Especially useful are derivatives of unsubstituted monocarboxylic acids with 2-6 carbon atoms, especially derivatives of acetic acid. In this context, acetyl starch, butyryl starch and propionyl starch are preferred.

[0036]   Furthermore, derivatives of unsubstituted dicarboxylic acids with 2-6 carbon atoms are preferred.

[0037]   In the case of derivatives of dicarboxylic acids, it is useful that the second carboxy group of the dicarboxylic acid is also esterified. Furthermore, derivatives of monoalkyl esters of dicarboxylic acids are also suitable in the context of the present invention.

[0038]   For the substituted mono- or dicarboxylic acids, the substitute groups may be preferably the same as mentioned above for substituted alkyl residues.

[0039]   Techniques for the esterification of starch are known in the art (see e.g. Klemm D. et al, Comprehensive Cellulose Chemistry Vol. 2, 1998, Whiley-VCH, Weinheim, New York, especially chapter 4.4, Esterification of Cellulose (ISBN 3-527-29489-9).

[0040]   According to a preferred embodiment of the present invention, hydroxyalkyl starch according to above-mentioned formula (I) is employed. The other saccharide ring structures comprised in HAS' may be the same as or different from the explicitly described saccharide ring, with the difference that they lack a reducing end.

[0041]   As far as the residues R_1, R_2 and R_3 according to formula (I) are concerned there are no specific limitations. According to a preferred embodiment, R_1, R_2 and R_3 are independently hydrogen or a hydroxyalkyl group, a hydroxyaralkyl group or a hydroxyalkaryl group having of from 2 to 10 carbon atoms in the respective alkyl residue. Hydrogen and hydroxyalkyl groups having of from 2 to 10 carbon atoms are preferred. More preferably, the hydroxyalkyl group has from 2 to 6 carbon atoms, more preferably from 2 to 4 carbon atoms, and even more preferably from 2 to 3 carbon atoms. In a preferred embodiment, hydroxyalkyl starch is hydroxyethyl starch in which R_1, R_2 and R_3 are independently hydrogen or a group $(CH_2CH_2O)_n$-H, wherein n is an integer, preferably 0, 1, 2, 3, 4, 5, or 6.

[0042]   "Hydroxyalkyl starch" therefore preferably comprises hydroxyethyl starch, hydroxypropyl starch and hydroxybutyl starch, wherein hydroxyethyl starch and hydroxypropyl starch are particularly preferred and hydroxyethyl starch is most preferred.

[0043]   The alkyl, aralkyl and/or alkaryl group may be linear or branched and suitably substituted.

[0044] Therefore, the present invention also relates to a method and a HAS derivative as described above wherein R_1, R_2 and R_3 are independently hydrogen or a linear or branched hydroxyalkyl group with from 2 to 6 carbon atoms.

[0045] Thus, R_1, R_2 and R_3 preferably may be H, hydroxyhexyl, hydroxypentyl, hydroxybutyl, hydroxypropyl such as 2-hydroxypropyl, 3-hydroxypropyl, 1-hydroxyisopropyl, hydroxyethyl such as 2-hydroxyethyl, hydrogen and the 2-hydroxyethyl group being especially preferred.

[0046] Therefore, the present invention also relates to a method and a HAS derivative as described above wherein R_1, R_2 and R_3 are independently hydrogen or a 2-hydroxyethyl group, an embodiment wherein at least one residue R_1, R_2 and R_3 being 2-hydroxyethyl being especially preferred.

[0047] Hydroxyethyl starch (HES) is most preferred for all embodiments of the present invention.

[0048] Therefore, the present invention relates to the method and a HAS derivative as described above, wherein the polymer is hydroxyethyl starch and the derivative is a hydroxyethyl starch (HES) derivative.

[0049] HAS, in particular HES, is mainly characterized by the molecular weight distribution, the degree of substitution and the ratio of C_2 : C_6 substitution. There are two possibilities of describing the substitution degree:

The degree of substitution (DS) of HAS is described relatively to the portion of substituted glucose monomers with respect to all glucose moieties.

[0050] The substitution pattern of HAS can also be described as the molar substitution (MS), wherein the number of hydroxyethyl groups per glucose moiety is counted.

[0051] In the context of the present invention, the substitution pattern of HAS, preferably HES, is referred to as MS, as described above (see also Sommermeyer et al., 1987, Krankenhauspharmazie, 8(8), 271-278, in particular p. 273).

[0052] MS is determined by Gas Chromatography after total hydrolysis of the HES molecule. MS values of respective HAS, in particular HES starting material are given. It is assumed that the MS value is not affected during the derivatization procedure in steps a) and b) of the process of the invention.

[0053] HAS and in particular HES solutions are present as polydisperse compositions, wherein each molecule differs from the other with respect to the polymerization degree, the number and pattern of branching sites, and the substitution pattern. HAS and in particular HES is therefore a mixture of compounds with different molecular weight. Consequently, a particular HAS and in particular HES solution is determined by average molecular weight with the help of statistical means. In this context, M_n is calculated as the arithmetic mean depending on the number of molecules. Alternatively, M_w (or MW), the weight average molecular weight, represents a unit which depends on the mass of the HAS, in particular HES.

[0054] In this context the number average molecular weight is defined by equation 1:

$$\overline{M}_n = \frac{\sum_i n_i \cdot M_i}{\sum_i n_i}$$

where n_i is the number of molecules of species i of molar mass M_i.

$\overline{M}_n$ indicates that the value is an average, but the line is normally omitted by convention.

[0055] M_w is the weight average molecular weight, defined by equation 2:

$$\overline{M}_w = \frac{\sum_i n_i \cdot M_i^2}{\sum_i n_i M_i}$$

where n_i is the number of molecules of species i of molar mass M_i

$\overline{M}_w$ indicates that the value is an average, but the line is normally omitted by convention.

[0056] Preferably, the hydroxyalkyl starch, in particular the hydroxyethyl starch, used in the invention has a mean molecular weight (weight mean) of from 1 to 500 kDa Hydroxyethyl starch can further exhibit a preferred molar substitution of from 0.1 to 3, preferably 0.1 to 2, more preferred 0.1 to 0.9 or 0.4 to 2, preferably 0.4 to 1.3, and a preferred ratio between C_2: C_6 substitution in the range of from 2 to 20 with respect to the hydroxyethyl groups.

[0057] The term "mean molecular weight" as used in the context of the present invention relates to the weight as determined according to the LALLS-(low angle laser light scattering)-GPC method as described in Sommermeyer et al., 1987, Krankenhauspharmazie, 8(8), 271-278; and Weidler et al., 1991, Arzneim.-Forschung/Drug Res., 41, 494-498. For mean molecular weights of 10 kDa and smaller, additionally, the calibration was carried out with a standard which had previously been qualified by LALLS-GPC.

[0058] According to a preferred embodiment of the present invention, the mean molecular weight of hydroxyethyl starch employed is from about 1 to about 500 kDa, more preferably from about 2 to 400 kDa, more preferably from about 5 to about 300 kDa, more preferably from about 10 to about 200 kDa, in particular from about 50 to about 150 kDa.

[0059] Further, the molar substitution of HAS and in particular HES is preferably from about 0.1 to about 3, preferably about 0.4 to about 1.3, such as 0.4, 0.5, 0.6, 0.7 0.8, 0.9, 1.0, 1.1, 1.2, or 1.3.

[0060] An example of HES having a mean molecular weight of about 5 to 300 kDa, preferably 50 to 150 kDa is a HES with a molar substitution of 0.1 to 3, preferably 0.4 to 1.3, such as 0.4, 0.5, 0.6, 0.7 0.8, 0.9, 1.0, 1.1, 1.2, or 1.3.

[0061] As far as the ratio of $C_2 : C_6$ substitution is concerned, said substitution is preferably in the range of from 2 to 20, more preferably in the range of from 2 to 15 and even more preferably in the range of from 3 to 12.

Other starches than hydroxyalkyl starches

[0062] In general, the methods of the present invention can also be carried out, and the derivatives of the present invention can also be prepared using other starches than hydroxyalkyl starches, in particular hydroxyethyl starch as described above, with the proviso that these starches also contain a reducing end being present in the hemiacetal form, optionally in equilibrium with the (free) aldehyde from, which reducing end may suitably be oxidised to give the respective oxidised form. In particular, a highly branched, unsubstituted or low-substituted starch product can be employed, i.e. a starch which has a significantly higher degree of branching than amylopectin and has the degree of alpha-1,6 branching of glycogen, or even exceeds this, and, if substituted, has a molar substitution MS of only up to 0.3, preferably of from 0.05 to 0.3. The term MS (molar substitution) as used in the context of this highly branched, unsubstituted or low-substituted starch product means the average number of hydroxyethyl or hydroxypropyl groups per anhydroglucose unit. The MS is normally measured by determining the content of hydroxyethyl or hydroxypropyl groups in a sample and computational allocation to the anhydroglucose units present therein. The MS can also be determined by gas chromatography. The degree of branching can be determined by a gas chromatographic methylation analysis as mol-% of the alpha-1,4,6-glycosidically linked anhydroglucoses in the polymer. The degree of branching is in every case an average because the highly branched, unsubstituted or low-substituted starch product of the invention is a polydisperse compound. The glucose units in said highly branched, unsubstituted or low-substituted starch product are linked via alpha-1,4- and alpha-1,6-linkages. The degree of branching means the proportion of alpha-1,4,6-linked glucose units in mol % of the totality of all anhydroglucoses. The C_2/C_6 ratio expresses the ratio or substitution at C-2 to that at C-6. The highly branched, unsubstituted or low-substituted starch product has a preferred degree of branching of from 6% to 50%, achievable by a transglucosidation step with the aid of branching enzymes. Even more preferably, the degree of branching is in the range of from 10 to 45, more preferably from 20 to 40 such as 20, 25, 30, 35, or 40. Also preferred are ranges of from more than 20 to 40, preferably from more than 20 to 30 such as from 21 to 40, preferably from 21 to 30. The starting material which can be used for this purpose is in principle any starch, but preferably waxy starches with a high proportion of amylopectin or the amylopectin fraction itself. The degree of branching which is necessary for the use according to the present invention of the starch products - as far as these "other starches" are concerned - is in the range from 8% to 20%, expressed as mol % of anhydroglucoses. This means that the starch products which can be used for the purposes of the invention have on average one alpha-1,6 linkage, and thus a branching point, every 12.5 to 5 glucose units. Preferred highly branched, unsubstituted or low-substituted starch products have a degree of branching of more than 10% and up to 20% and in particular from 11 to 18%. A higher degree of branching means a greater solubility of the starch products of the invention and a greater bioavailability of these dissolved starch products in the body. Particular preference is given to unmodified starch products with a degree of branching of more than 10%, in particular from 11% to 18%. The highly branched, unsubstituted or low-substituted starch product can be prepared by targeted enzymatic assembly using so-called branching or transfer enzymes, where appropriate followed by partial derivatisation of free hydroxyl groups with hydroxyethyl or hydroxypropyl groups. Instead of this it is possible to convert a hydroxyethylated or hydroxypropylated starch by enzymatic assembly using so-called branching or transfer enzymes into a highly branched, unsubstituted or low-substituted starch product. Obtaining branched starch products enzymatically from wheat starch with a degree of branching of up to 10% is known per se and described for example in WO 00/66633 A. Suitable branching or transfer enzymes and the obtaining thereof are disclosed in WO 00/18893 A, US 4,454,161, EP 0 418 945 A, JP 2001294601 A or US 2002/065410 A. This latter publication describes unmodified starch products with degrees of branching of more than 4% and up to 10% or higher. The enzymatic transglycosilation can be carried out in a manner known per se, for example by incubating waxy corn starch, potato starch obtained from potatoes having a high amylopectin content, or starch obtained from rice, from manioc, from wheat, from wheat having a high amylopectin
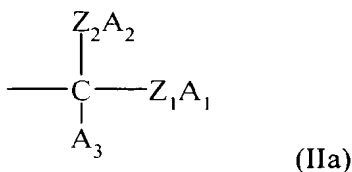
content, from corn, from corn having a high amolypectin content, or from corn having a high amylose content, with the appropriate enzymes under mild conditions at pH values between 6 and 8 and temperatures between 25 and 40 °C in aqueous solution. The molecular weight M_w means, as used in the context of the highly branched, unsubstituted or low-substituted starch products, the weight average molecular weight. This can be determined in a manner known per se by various methods, i.e. by gel permeation chromatography (GPC) or high pressure liquid chromatography (HPLC) in conjunction with light scattering and RI detection. The C_2/C_6 ratio preferred for substituted starches is in the range from 5 to 9. The high degree of branching of the highly branched, unsubstituted or low-substituted starch products increases the solubility in water thereof to such an extent that hydroxyethyl or hydroxypropyl substitution can be wholly or substantially dispensed with in order to keep the starch product in solution. The average molecular weight of the highly branched, unsubstituted or low-substituted starch product can be increased in a suitable manner via the permeability limit of the peritoneum. The characteristic variable which can be used in this case is also the GPC value of the so-called bottom fraction BF90% (molecular weight at 90% of the peak area as a measure of the proportion of smaller molecule fractions). A greater ultrafiltration (UF) efficiency can be achieved by appropriate raising of the molecular weight with, at the same time, a drastically reduced absorption across the peritoneal membrane. At the same time, high molecular weight residual fragments which are produced by degradation by endogenous amylase, which can no longer be further degraded by amylase, and which are stored in organs or tissues, no longer occur or now occur to only a slight extent.

[0063] According to the present invention, hydroxyalkyl starch is reacted with a crosslinking compound M-L-A wherein M is an amino group and A is an acetal group or a ketal group, group M and group L being separated by a suitable spacer.

The acetal or ketal group A

[0064] As far as the acetal group or ketal group A is concerned, no specific limitations exist. In the context of the present invention, the term "acetal group" also comprises sulphur acetals and nitrogen acetals, and the term "ketal group" also comprises also sulphur ketals and nitrogen ketals. Additionally, as far as the term "acetal group" is concerned, hemiacetals are explicitly excluded, and as far as the term "ketal group" is concerned, hemiketals are explicitly excluded.

[0065] According to a preferred embodiment of the present invention, group A of the crosslinking compound M-L-A is a residue according to formula (IIa)

$$\begin{array}{c} Z_2A_2 \\ | \\ ---C---Z_1A_1 \\ | \\ A_3 \end{array} \qquad \text{(IIa)}$$

wherein
Z_1 and Z_2 are each independently O or S or NR_x, preferably O, wherein R_x is H or lower alkyl such as methyl, ethyl, or propyl such as n-propyl or i-propyl, or $C(O)$-R_y wherein R_y is preferably selected from the group consisting of C_1-C_6 alkyl and C_6-C_{14} aryl, even more preferably selected from the group consisting of optionally substituted, preferably non-substituted methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, and tert-butyl; R_x preferably being H;
A_1 and A_2 are each independently methyl, ethyl, n-propyl, isopropyl, n-butyl, tert-butyl, benzyl, 1,1,1-trichloroethyl, nitrobenzyl, methoxybenzyl, ethoxybenzyl, or are forming a ring according to formula (IIb)

$$\begin{array}{c} Z_2A_2 \\ | \quad \diagdown \\ ---C---Z_1A_1 \\ | \\ A_3 \end{array} \qquad \text{(IIb)}$$

wherein A_1 and A_2, taken together, are -$(CH_2)_2$- or -$(CH_2)_3$- or -$(CH_2CH(CH_3))$-, and wherein A_3 is H or methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, pentyl, benzyl, or is forming a ring with the N atom of the amino group M or with a suitable atom comprised in L.
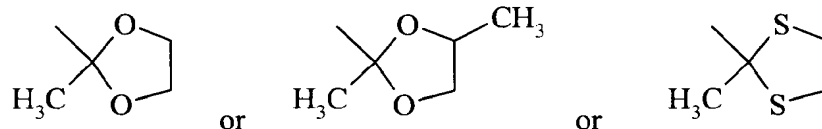
[0066] Preferably, at least one of Z_1 and Z_2 is O, more preferably both Z_1 and Z_2 are O.

[0067] As far as the residue A_3 is concerned, acetal groups are preferred according to the present invention, i.e. A_3 is preferably H.

[0068] If A is a ketal group, it is preferred that A_3 is methyl. Therefore, conceivable ketal groups A according to the present invention are, among others,
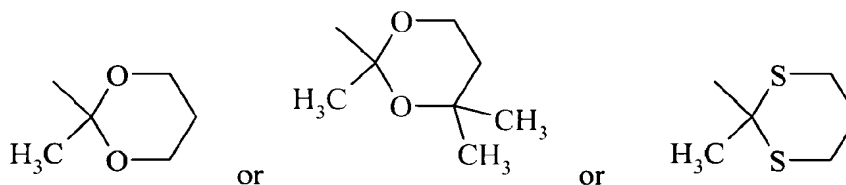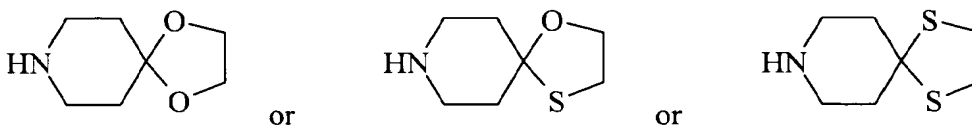
or

or

[0069] If A_3 is, e.g., forming a ring with either the N atom of the amino group M or with a suitable atom comprised in L, conceivable crosslinking compounds according to the present invention are, e.g.,

or

or

An especially preferred crosslinking compound according to the present invention is
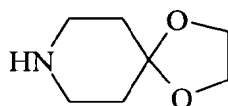
i.e., the amino group M is a secondary amine, both Z_1 and Z_2 are O, and A_1 and A_2, taken together, are -$(CH_2)_2$-.

[0070] According to a preferred embodiment, A_1 and A_2 are each methyl or ethyl, even more preferably ethyl. Therefore, a particularly preferred acetal group A according to the present invention is -$CH(OCH_3)_2$ or -$CH(OC_2H_5)_2$, in particular -$CH(OC_2H_5)_2$.

[0071] According to a further embodiment wherein A_1 and A_2 are forming a ring according to formula (IIb), A_1 and A_2, taken together, are preferably -$(CH_2)_2$-. As far as this embodiment is concerned, particularly preferred acetal groups A according to the present invention are

and
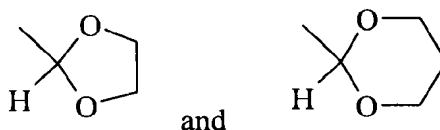
[0072] The amino group M

[0073] As far as the amino group M is concerned, no particular restrictions exist with the proviso that the amino group can be reacted with either the oxidised or non-oxidised reducing end, i.e. via carbon atom C* of the reducing terminal saccharide unit of HAS, preferably HES, in either the non-oxidised state, i.e. as hemiacetal or as free aldehyde group, or in the oxidised state, i.e. as lactone or as free carboxy group. The term "amino group" as used in this context of the present application also comprises suitable salts of the amino group, such as, e.g., protonated amino groups, with a pharmaceutically acceptable anion, such as, e.g., chloride, hydrogen sulfate, sulfate, carbonate, hydrogen carbonate, citrate, phosphate, or hydrogen phosphate.

[0074] Preferably, the amino group of the crosslinking compound M-L-A according to the present invention is a group according to formula (IIc)

$$\text{R'} - \underset{\underset{H}{|}}{N} - Y -$$

wherein Y is either absent or is a chemical moiety selected from the group consisting of
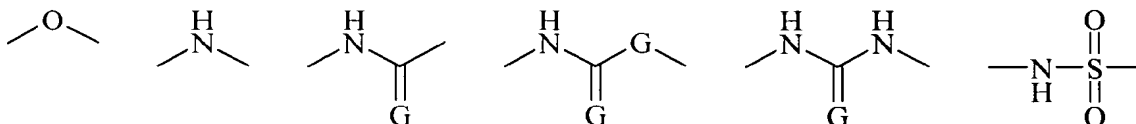
wherein G is O or S or NH, and, if present twice, each G is independently O or S or NH, G preferably being O, and wherein R' is H or a hydroxy group or an organic residue selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, alkylaryl, and substituted alkylaryl. In this context, the term "alkyl" relates to non-branched alkyl residues, branched alkyl residues, and cycloalkyl residues. Preferably, each of these organic residues has from 1 to 10 carbon atoms. As conceivable substituents, halogens such as F, Cl or Br may be mentioned. Preferably, the organic residues are non-substituted hydrocarbons.

[0075] If R' is a hydroxy group, the preferred amino group of the present invention is HO-NH-, i.e. Y is absent.

[0076] Preferably, in case R' is an organic residue, R' is selected from the group consisting of alkyl and substituted alkyl, the alkyl residue being especially preferred. Even more preferably, the optionally substituted alkyl residue has from 1 to 10, more preferably from 1 to 6, more preferably from 1 to 4 such as 1, 2, 3, or 4 carbon atoms. Thus, preferred organic residues according to the present invention are methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, or t-butyl. According to an especially preferred embodiment, the organic residue R' is methyl or ethyl, in particular methyl.

[0077] Therefore, in case R' is an organic residue, preferred amino groups according to the present invention are, e.g., $H_3C\text{-}CH_2\text{-}NH\text{-}$, $H_3C\text{-}NH\text{-}$, $H_3C\text{-}CH_2\text{-}NH\text{-}O\text{-}$, and $H_3C\text{-}NH\text{-}O\text{-}$, with $H_3C\text{-}NH\text{-}$ and $H_3C\text{-}NH\text{-}O\text{-}$ being particularly preferred.

[0078] According to the present invention, it is also possible that R' is not a separate residue but forms a ring structure with a suitable atom comprised in L or with residue A_3 of group A of the crosslinking compound. These structures are also comprised in above-mentioned definition of the term "alkyl" with respect to R'. By way of example, R' can form a ring structure with residue A_3 of group A of the crosslinking compound, A being a ketal group. Conceivable crosslinking compounds are, e.g.,
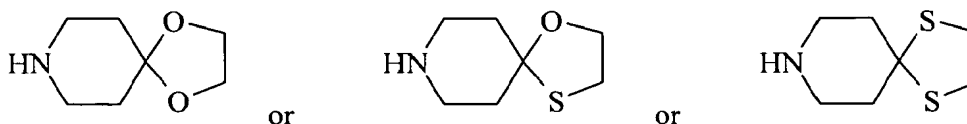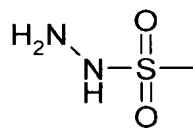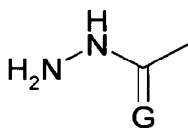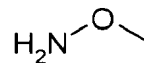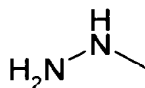

[0079] In this case, an especially preferred crosslinking compound according to the present invention is
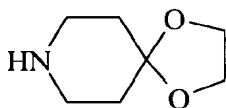
i.e., the amino group M is a secondary amine, both Z_1 and Z_2 are O, and A_1 and A_2, taken together, are $-(CH_2)_2-$.

[0080]   In a preferred embodiment of the present invention, R' is H. Thus, preferred amino groups M of the present are
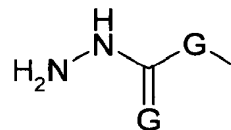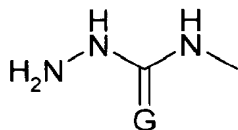
wherein G is O or S, and, if present twice, independently O or S, O being preferred.

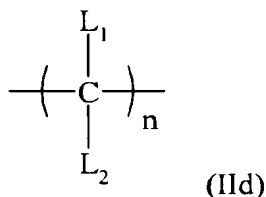[0081]   Especially preferred amino groups M of the present invention, if R' is H, are H_2N-, H_2N-O-, and $H_2N-NH-(C=O)-$.

[0082]   Hence, the present invention also relates to the method and the derivative mentioned above, wherein the amino group M is H_2N-, H_2N-O-, $H_2N-NH-(C=O)-$, H_3C-NH- or $H_3C-NH-O-$, preferably H_2N-, H_2N-O-, or $H_2N-NH-(C=O)-$.

The spacer L

[0083]   According to the present invention, functional groups M and A of the crosslinking compound are separated by a suitable spacer. The term "spacer" as used in this context of the present application relates to any suitable chemical moiety bridging M and A.

[0084]   In general, there no particular restrictions as to the chemical nature of the spacer L with the proviso that L has in particular chemical properties enabling carrying out the inventive method for the preparation of the novel derivatives and providing suitable chemical properties for the novel derivatives as far as their intended uses is concerned.

[0085]   According to a preferred embodiment of the present invention, L bridging M and A is a spacer comprising at least one structural unit according to formula (IId)

wherein L_1 and L_2 are independently from each other H or an organic residue selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, alkylaryl, substituted alkylaryl, and residues -O-R" wherein R" is selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, arylalkyl, substituted

arylalkyl, alkylaryl, substituted alkylaryl.

[0086] In this context, the term "alkyl" relates to non-branched alkyl residues, branched alkyl residues, and cycloalkyl residues. As preferred substituents, halogens such as F, Cl or Br may be mentioned.

[0087] Preferably, L_1 and L_2 are independently from each other H or an organic residue selected from the group consisting of alkyl and substituted alkyl; more preferably, L_1 and L_2 are independently from each other H or alkyl; even more preferably, both L_1 and L_2 are H.

[0088] Preferably, if L_1 and L_2 are organic residues, each of L_1 and L_2 may independently contain 1 to 20, preferably 1 to 10, more preferably 1 to 6, more preferably 1 to 6, more preferably 1 to 4 carbon atoms. Especially preferred are residues L_1 and L_2 such as optionally substituted, preferably non-substituted methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, tert-butyl residues. According to the present invention, L_1 may be H and L_2 may be an organic residue as defined above.

[0089] As far as integer n is concerned, n is preferably from 1 to 20, preferably from 1 to 10, more preferably from 1 to 6, more preferably from 1 to 4, more preferably 2.

[0090] If integer n is greater than 1, the groups (CL_1L_2) may be the same or different from each other. According to a preferred embodiment of the present invention, groups (CL_1L_2) directly linked to each other have the same constitution.

[0091] According to a preferred embodiment of the present invention, the spacer L consists of a structural unit according to formula (IId) wherein L_1 and L_2 are as defined above. More preferably, integer n is from 1 to 20, more preferably from 1 to 10 such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Even more preferably, each of the groups (CL_1L_2) is (CH_2) such that spacer L bridging M and A has the structure
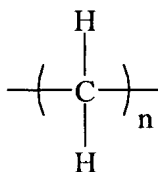
wherein n is an integer from 1 to 20, more preferably from 1 to 10 such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Even more preferably, n is in the range of from 1 to 6, more preferably in the range of from 1 to 4, such as 1, 2, 3, or 4, and in particular 2.

[0092] Therefore, according to a particularly preferred embodiment of the present invention, spacer L is $-CH_2-CH_2-$.

[0093] According to further embodiments of the present invention, spacer L of the crosslinking compound comprises at least one structural unit according to formula (IId)

(IId)
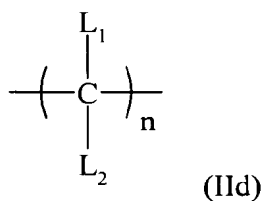
wherein n, L_1 and L_2 are as defined above, preferably
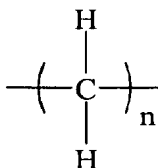
and wherein L further comprises at least one chemical moiety different from $-(CL_1L_2)-$.

[0094] In this context, embodiments may be mentioned wherein the spacer L consists of a structural unit $-(CL_1L_2)_n-$ and a further chemical moiety T which separates $-(CL_1L_2)_n-$ from group M or A. Also embodiments are conceivable wherein the spacer L consists of a structural unit $-(CL_1L_2)_n-$ and two further chemical moieties T_1 and T_2 wherein T_1 separates $-(CL_1L_2)_n-$ from group M and T_2 separates $-(CL_1L_2)_n-$ from group A. Thus, the present invention also encom-

passes embodiments according to which the crosslinking compound has one of the following structures:

$$M\text{-}T\text{-}(CL_1L_2)_n\text{-}A$$

$$M\text{-}(CL_1L_2)_n\text{-}T\text{-}A$$

$$M\text{-}T_1\text{-}(CL_1L_2)_n\text{-}T_2\text{-}A$$
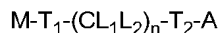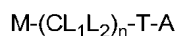
[0095] As to the chemical moieties T, T_1 or T_2, there are no particular restrictions as to their chemical nature with the proviso that L has in particular chemical properties enabling carrying out the inventive method for the preparation of the novel derivatives and providing suitable chemical properties for the novel derivatives as far as their intended uses is concerned.

[0096] Therefore, T, T_1 and/or T_2 may comprise optionally substituted aryl residues, suitable heteroatoms, suitable functional groups, or the like. As far as functional groups are concerned, embodiments may be mentioned according to which these functional groups result from the preparation of the crosslinking compound wherein at least a first compound and at least a second compound are reacted with each other to give a compound M-L-A. By way of example, a first compound $M\text{-}L'\text{-}W_1$ and a second compound $W_2\text{-}L''\text{-}A$ may be reacted to give crosslinking compound M-L-A wherein -L- is -L'-F-L''-A and F represents the functional group resulting from the reaction of functional group W_1 with functional group W_2, and wherein at least one of L' and L'' comprises the structure unit $-(CL_1L_2)n-$. Such functional groups W_1 and W_2 may be suitably chosen. By way of example, one of groups W_1 and W_2, i.e. W_1 or W_2, may be chosen from the group consisting of the functional groups according to the following list while the other group, W_2 or W_1, is suitable selected and capable of forming a chemical linkage with W_1 or W_2, wherein W_2 or W_1 is also preferably selected from the above-mentioned group:

- C-C-double bonds or C-C-triple bonds or aromatic C-C-bonds;
- the thio group or the hydroxy groups;
- alkyl sulfonic acid hydrazide, aryl sulfonic acid hydrazide;
- 1,2-dioles;
- 1,2 amino-thioalcohols;
- azides;
- 1,2-aminoalcohols;
- the amino group $-NH_2$ or derivatives of the amino groups comprising the structure unit -NH- such as aminoalkyl groups, aminoaryl group, aminoaralkyl groups, or alkarlyaminogroups;
- the hydroxylamino group $-O\text{-}NH_2$, or derivatives of the hydroxylamino group comprising the structure unit -O-NH-, such as hydroxylalkylamino groups, hydroxylarylamino groups, hydroxylaralkylamino groups, or hydroxalalkarylamino groups;
- alkoxyamino groups, aryloxyamino groups, aralkyloxyamino groups, or alkaryloxyamino groups, each comprising the structure unit -NH-O-;
- residues having a carbonyl group, -Q-C(=G)-M', wherein G is O or S, and M' is, for example,

  - -- -OH or -SH;
  - -- an alkoxy group, an aryloxy group, an aralkyloxy group, or an alkaryloxy group;
  - -- an alkylthio group, an arylthio group, an aralkylthio group, or an alkarylthio group;
  - -- an alkylcarbonyloxy group, an arylcarbonyloxy group, an aralkylcarbonyloxy group, an alkarylcarbonyloxy group;
  - -- activated esters such as esters of hydroxylamines having imid structure such as N-hydroxysuccinimid;

- $-NH\text{-}NH_2$, or -NH-NH-;
- $-NO_2$;
- the nitril group;
- carbonyl groups such as the aldehyde group or the keto group;
- the carboxy group;
- the -N=C=O group or the -N=C=S group;
- vinyl halide groups such as the vinyl iodide or the vinyl bromide group or triflate;
- $-C{\equiv}C\text{-}H$;
- $-(C{=}NH_2Cl)\text{-}OAlkyl$
- groups $-(C{=}O)\text{-}CH_2\text{-}Hal$ wherein Hal is Cl, Br, or I;
- $-CH{=}CH\text{-}SO_2\text{-}$;

- a disulfide group comprising the structure -S-S-;
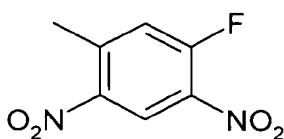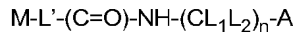- the group

;

- the group

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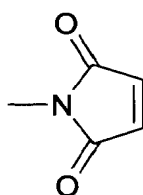
[0097] By way of example, W_1 or W_2 may be a carboxy group or an activated ester, and W_2 or W_1 may be an amino group or a hydroxy group such that F representing the functional group resulting from the reaction of functional group W_1 with functional group W_2, is an amid or an ester.
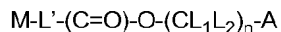
[0098] Therefore, by way of example, crosslinking compounds having a spacer L comprising, apart from structure unit $-(CL_1L_2)_n-$, a functional group, may have a constitution such as

$$M\text{-}L'\text{-}(C{=}O)\text{-}NH\text{-}(CL_1L_2)_n\text{-}A$$

or

$$M\text{-}L'\text{-}(C{=}O)\text{-}O\text{-}(CL_1L_2)_n\text{-}A$$

or

$$M\text{-}L'\text{-}NH\text{-}(C{=}O)\text{-}(CL_1L_2)_n\text{-}A$$

or

$$M\text{-}L'\text{-}O\text{-}(C{=}O)\text{ -}(CL_1L_2)_n\text{-}A$$

or

$$M\text{-}(CL_1L_2)_n\text{-}(C{=}O)\text{-}NH\text{-}L''\text{-}A$$

or

$$M\text{-}(CL_1L_2)_n\text{-}(C{=}O)\text{-}O\text{-}L''\text{-}A$$

or

$$M\text{-}(CL_1L_2)_n\text{-}NH\text{-}(C{=}O)\text{-}L''\text{-}A$$

or

$$M\text{-}(CL_1L_2)_n\text{-}O\text{-}(C{=}O)\text{-}L''\text{-}A$$
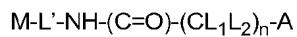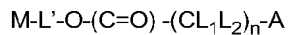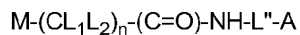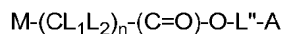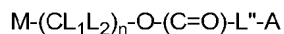
wherein L' and L" may or may not comprise a structure unit $-(CL_1L_2)_n-$.

**[0099]** Again by way of example and in order to illustrate above-discussed structures, a crosslinking compound conceivable in the context of the present invention may be


or

or
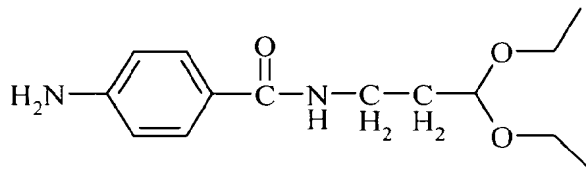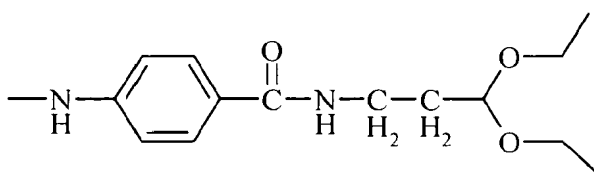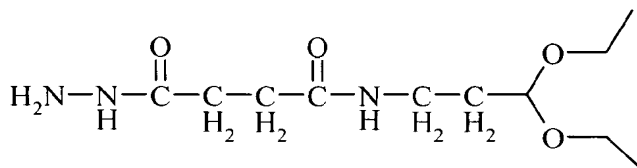
[0100] According to a further embodiment of the present invention, spacer L may comprise more than one structure units $-(CL_1L_2)_n-$ wherein these structure units may be same or different, i.e. the structure may differ in n and/or L_1 and/or L_2, wherein at least two such structure units may be separated by a heteroatom such as O or S. Preferably, according to this embodiment, the spacer L comprises at least one structure unit $-(CL_1L_2)_{n1}-O-(CL_1L_2)_{n2}-$, preferably $-(CH_2)_{n1}-O-(CH_2)_{n2}$ wherein n1 is equal to or different from n2, and wherein the spacer L is linked via $-(CL_1L_2)_{n1}-$ to the amino group M of the crosslinking compound, i.e. the crosslinking compound comprising the following sub-structure

$$M-(CL_1L_2)_{n1}-O-(CL_1L_2)_{n2}-$$
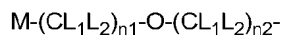
[0101] According to a preferred conceivable embodiment, spacer structures such as

$$-((CL_1L_2)_{n1}-)O)_m-(CL_1L_2)_{n2}-$$
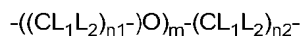
may be mentioned, with m being an integer from 1 to 20, preferably from 1 to 10, more preferably from 1 to 6 such as 1, 2, 3, 4, 5, or 6. More preferably n1 is from 2 to 4, and most preferably 2. Preferably, n2 is from 1 to 4, more preferably 1 or 2. Therefore, preferred structures are, by way of example,

$$-((CL_1L_2)_2-O)_m-(CL_1L_2)-$$

and more preferably

$$-((CH_2)_2-O)_m-CH_2-$$
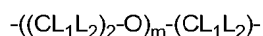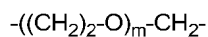
[0102] Again by way of example and in order to illustrate above-discussed structures, a crosslinking compound conceivable in the context of the present invention may be

or
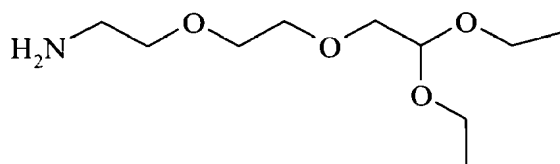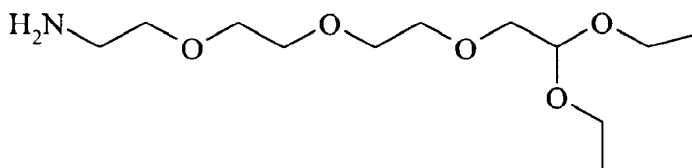
[0103] According to a further embodiment of the present invention, group M and group A may be separated by 2 suitable chemical moieties, at least one thereof comprising $-(CL_1L_2)n-$, such that the N atom of group M and the C atom of ketal group A are forming a ring. A preferred embodiment was already presented above and has the structure
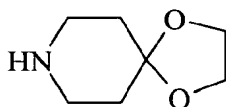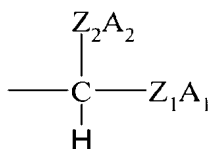

[0104] Especially preferred crosslinking compounds of the present invention are compounds having, as group M, the group H_2N- or the group H_2N-O- or the group $H_2N-NH-(C=O)-$, especially preferably the group H_2N-, and, as group A, an acetal group, preferably an acetal group having the structure

wherein, more preferably, Z_1 and Z_2 are O and, even more preferably, A_1 and A_2 are both ethyl. Even more preferably, the spacer L consists of structure unit $-(CL_1L_2)_n-$, with L_1 and L_2 preferably being H, and integer n even more preferably being from 1 to 20, preferably from 1 to 10, more preferably from 1 to 6, more preferably from 1 to 4, more preferably 2.
[0105] Therefore, preferred crosslinking compounds according to the present invention are, by way of example,
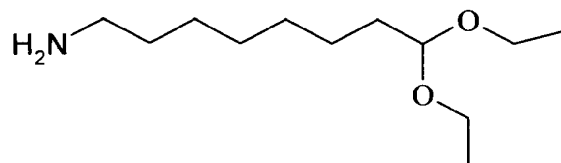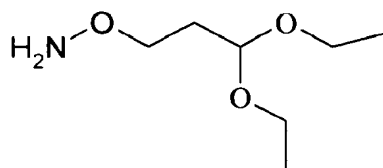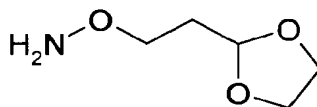
or

or

or

or
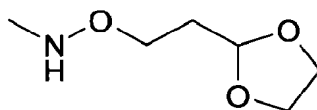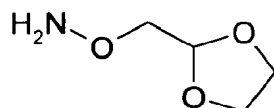

or

or
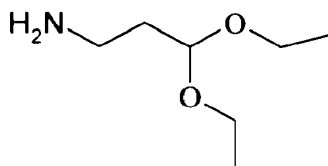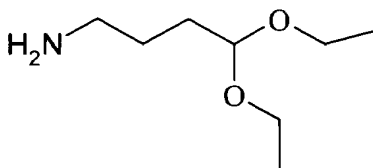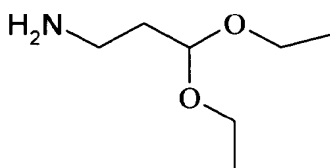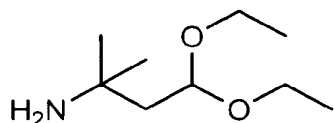

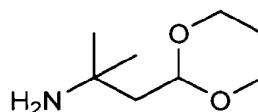[0106]   Particularly preferred as crosslinking compound M-L-A is 1-amino-3,3-diethoxypropane,

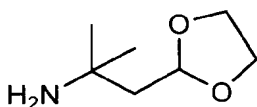[0107] By way of example, conceivable amino-acetal crosslinking compounds according to the present invention are:
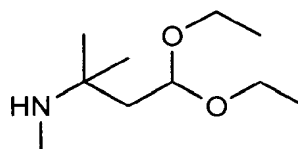
4,4-diethoxy-2-methylbutan-2-amine
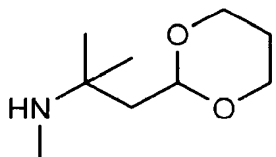
1-(1,3-dioxan-2-yl)-2-methylpropan-2-amine
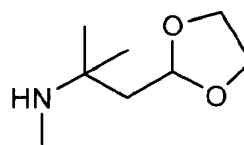
1-(1,3-dioxolan-2-yl)-2-methylpropan-2-amine
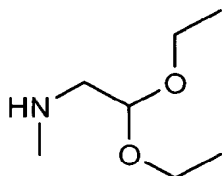
4,4-diethoxy-*N*,2-dimethylbutan-2-amine
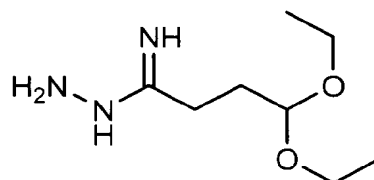
1-(1,3-dioxan-2-yl)-*N*,2-dimethylpropan-2-amine

1-(1,3-dioxolan-2-yl)-*N*,2-dimethylpropan-2-amine

2,2-diethoxy-*N*-methylethanamine

4,4-diethoxybutanimidhydrazide
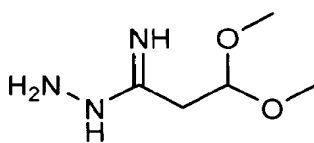
3,3-dimethoxypropanimidhydrazide

[0108] By way of example, conceivable amino-ketal crosslinking compounds according to the present invention are:
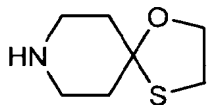
1-oxa-4-thia-8-azaspiro[4.5]decane
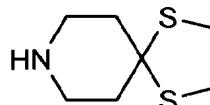
1,4-dithia-8-azaspiro[4.5]decane
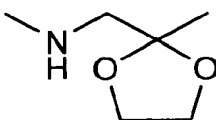
N-methyl-1-(2-methyl-1,3-dioxolan-2-yl)
methanamine

1-(3,4-dimethoxyphenyl)-2-(2-methyl-
1,3-dioxolan-2-yl)ethanamine
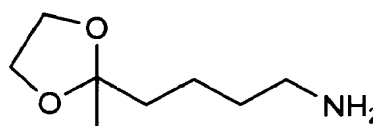
4-(2-methyl-1,3-dioxolan-2-yl)butan-1-amine

1-(2-methyl-1,3-dioxolan-2-yl)hexan-2-amine
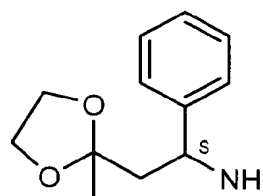
2-(2-methyl-1,3-dioxolan-2-yl)-1-
phenylethanamine
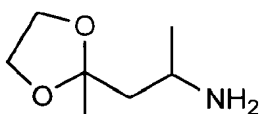
1-(2-methyl-1,3-dioxolan-2-yl)propan-2-amine
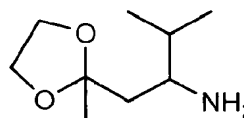
3-methyl-1-(2-methyl-1,3-dioxolan-2-yl)butan-2-amine
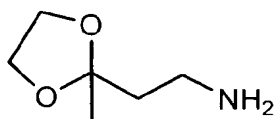
2-(2-methyl-1,3-dioxolan-2-yl)ethanamine
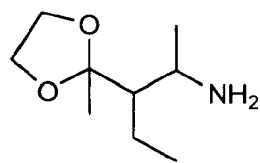
3-(2-methyl-1,3-dioxolan-2-yl)pentan-2-amine
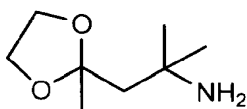
2-methyl-1-(2-methyl-1,3-dioxolan-2-yl)propan-2-amine
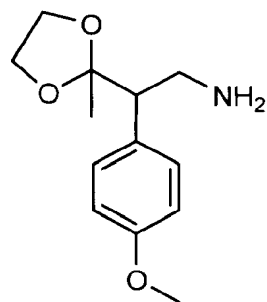
2-(4-methoxyphenyl)-2-(2-methyl-
1,3-dioxolan-2-yl)ethanamine
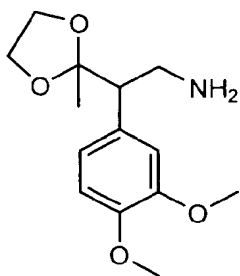
2-(3,4-dimethoxyphenyl)-2-(2-methyl-
1,3-dioxolan-2-yl)ethanamine
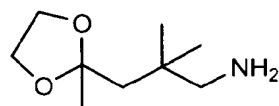
2,2-dimethyl-3-(2-methyl-1,3-dioxolan-
2-yl)propan-1-amine
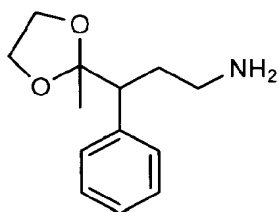
3-(2-methyl-1,3-dioxolan-2-yl)-3-
phenylpropan-1-amine
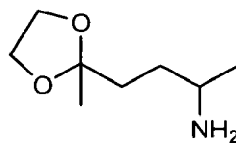
4-(2-methyl-1,3-dioxolan-2-yl)butan-2-amine
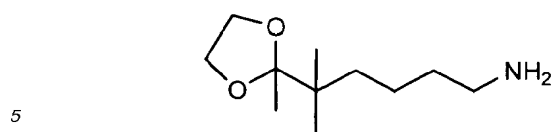
5-methyl-5-(2-methyl-1,3-dioxolan-
2-yl)hexan-1-amine
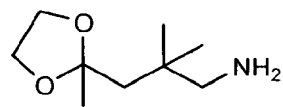
2,2-dimethyl-3-(2-methyl-1,3-dioxolan-2-yl)propan-1-amine
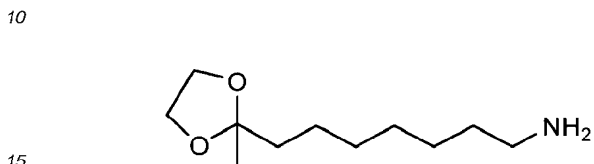
7-(2-methyl-1,3-dioxolan-2-yl)heptan-1-amine
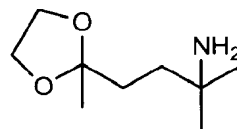
2-methyl-4-(2-methyl-1,3-dioxolan-2-yl)butan-2-amine
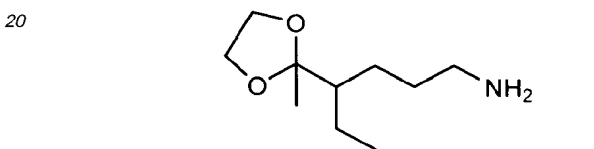
4-(2-methyl-1,3-dioxolan-2-yl)hexan-1-amine
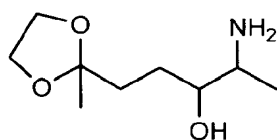
4-amino-1-(2-methyl-1,3-dioxolan-2-yl)pentan-3-ol
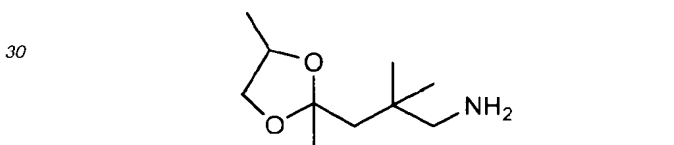
3-(2,4-dimethyl-1,3-dioxolan-2-yl)-2,2-dimethylpropan-1-amine
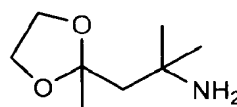
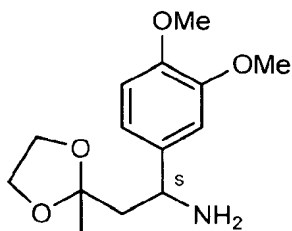2-methyl-1-(2-methyl-1,3-dioxolan-2-yl)propan-2-amine
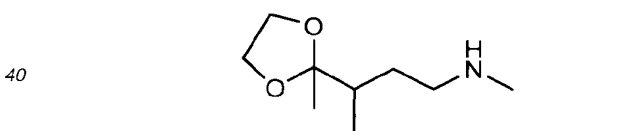
N-methyl-3-(2-methyl-1,3-dioxolan-2-yl)butan-1-amine
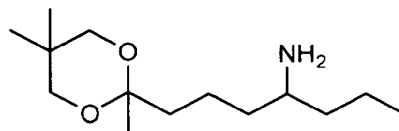
1-(2,5,5-trimethyl-1,3-dioxan-2-yl)heptan-4-amine
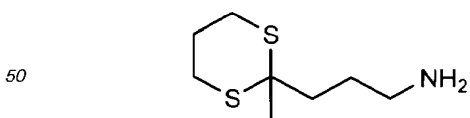
3-(2-methyl-1,3-dithian-2-yl)propan-1-amine
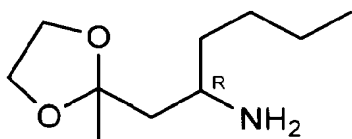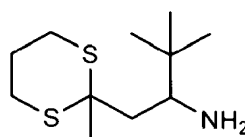
3,3-dimethyl-1-(2-methyl-1,3-dithian-2-yl)butan-2-amine
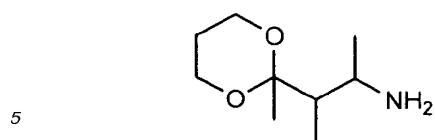
3-(2-methyl-1,3-dioxan-2-yl)butan-2-amine
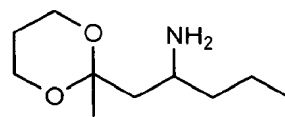
1-(2-methyl-1,3-dioxan-2-yl)pentan-2-amine
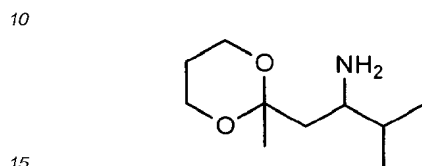
3-methyl-1-(2-methyl-1,3-dioxan-2-yl)butan-2-amine
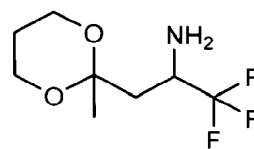
1,1,1-trifluoro-3-(2-methyl-1,3-dioxan-2-yl)propan-2-amine

Hydroxyalkyl starch derivative

[0109]   Accordingly, the present invention relates to a hydroxyalkyl starch (HAS) derivative obtainable or obtained by the method as described above.

[0110]   Moreover, the present invention relates to a HAS derivative of formula (III)
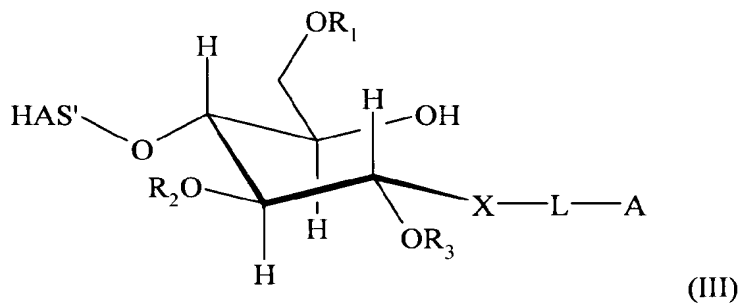
$$(III)$$

wherein A is an acetal or ketal group; L is a spacer bridging X and A;
wherein X is the functional group resulting from the reaction of an amino group M of a crosslinking compound of formula (II)

M-L-A

with hydroxyalkyl starch (HAS) of formula (I)

$$(I)$$
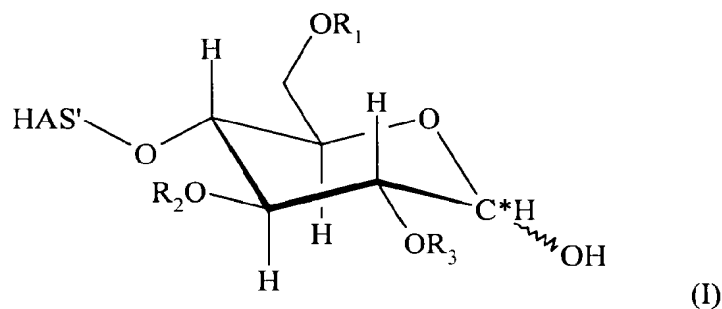
via carbon atom C* of the HAS, wherein C* is optionally oxidised prior to the reaction of HAS with M,
wherein HAS' is the remainder of the hydroxyalkyl starch molecule and R_1, R_2 and R_3 are independently hydrogen or
a linear or branched hydroxyalkyl group.

[0111] As far as preferred embodiments regarding HAS, preferably HES, L, A, R_1, R_2, and R_3 are concerned, specific
reference is made to the embodiments as described hereinabove.

[0112] Further, the present invention relates to the HAS derivative as described above, wherein R_1, R_2 and R_3 are
independently a group -$(CH_2CH_2O)_n$-H, wherein n is an integer, preferably 0, 1, 2, 3, 4, 5, or 6.

[0113] Further, the present invention relates to the HAS derivative as described above, wherein the hydroxyalkyl starch
is hydroxyethyl starch (HES).

[0114] Further, the present invention relates to the HAS derivative as described above, wherein A is a residue according
to formula (IIa)
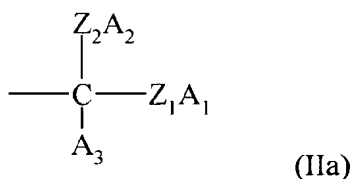
$$\overset{\displaystyle Z_2A_2}{\underset{\displaystyle A_3}{\overset{\displaystyle |}{\underset{\displaystyle |}{-\!\!-\!\!C-\!\!-Z_1A_1}}}}$$

(IIa)

wherein

Z_1 and Z_2 are each independently O or S or NR_x, preferably O, wherein R_x is H or lower alkyl such as methyl, ethyl,
or propyl, preferably H;
A_1 and A_2 are each independently methyl, ethyl, n-propyl, isopropyl, n-butyl, tert-butyl, benzyl, 1,1,1-trichloroethyl,
nitrobenzyl, methoxybenzyl, ethoxybenzyl, or are forming a ring according to formula (IIb)

$$\overset{\displaystyle Z_2A_2}{\underset{\displaystyle A_3}{\overset{\displaystyle |}{\underset{\displaystyle |}{-\!\!-\!\!C-\!\!-Z_1A_1}}}}$$

(IIb)
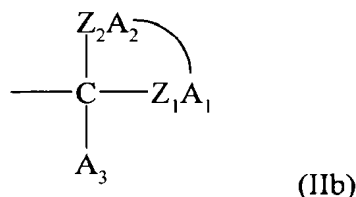
wherein A_1 and A_2, taken together, are -$(CH_2)_2$- or -$(CH_2)_3$- or -$(CH_2CH(CH_3))$-, and wherein A_3 is H or methyl, ethyl,
n-propyl, isopropyl, n-butyl, isobutyl, pentyl, benzyl, or is forming a ring with the N atom of the amino group M or with a
suitable atom comprised in L, A_3 preferably being H.

[0115] The precise chemical nature of group X of the HAS derivative according to the invention depends on the
respective chemical nature of group M, on the oxidation state of the carbon atom C* of the reducing end of HAS, and
on the reaction conditions such as solvent, temperature and so forth employed for the reaction. According to embodiments
of the present invention wherein the carbon atom C* is employed in oxidised and non-oxidised state, specific and preferred
examples are discussed in detail hereinunder.

[0116] Preferably, as far as X is concerned, the present invention relates to the HAS derivative as described above,
wherein X is selected from the group consisting of -CH=N-, -CH_2-NH-, -CH=N-O-, -CH_2-NH-O-, -C(=O)-NH-, -C(=O)-NH-
NH-, -CH=NH-NH-(C=O)-, -CH_2-NH-NH-(C=O)-, preferably consisting of -CH_2-NH-, -CH=N-, -CH=N-O-, -CH_2-NH-O-,
-CH=N-NH-(C=O)-, and -CH_2-NH-NH-(C=O)-, more preferably consisting of -CH_2-NH-, -CH=N-, -CH=N-O-, and
-CH_2-NH-O-.

[0117] For certain embodiments of the group X, it is conceivable that the terminal saccharide unit of the HAS as present
in the HAS derivative is present in a ring structure which may be in equilibrium with the open structure according to
formula (III) above, the ring structure and the open structure having a certain equilibrium distribution. In these cases,
and for the purpose of the present invention, formula (III) as given above comprises the open structure as well as the
ring structure, and formula (III) does not restrict the HAS derivative to the open structure. For specific and preferred
examples are discussed in detail hereinunder, the ring structure is shown in some cases.

[0118] Further, the present invention relates to the HAS derivative as described above, wherein L bridging M and A
is a spacer comprising at least one structural unit according to formula (IId), preferably consisting of a structural unit

according to formula (IId)

$$-\left(\begin{array}{c} L_1 \\ | \\ C \\ | \\ L_2 \end{array}\right)_n \quad \text{(IId)}$$

wherein L_1 and L_2 are independently from each other H or an organic residue selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, alkylaryl, substituted alkylaryl, and residues -O-R" wherein R" is selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, alkylaryl, substituted alkylaryl; preferably H or an organic residue selected from the group consisting of alkyl and substituted alkyl; more preferably H or alkyl; more preferably H,

wherein n is an integer from 1 to 20, preferably from 1 to 10, more preferably from 1 to 6, more preferably from 1 to 4, more preferably 2.

The optionally oxidised reducing end of HAS, preferably HES

[0119] According to the present invention, HAS, preferably HES can be reacted via carbon atom C^* of the terminal reducing end of the starch with amino group M of the crosslinking compound wherein C^* is optionally oxidised prior to the reaction of HAS with M.

[0120] The term "the HAS is reacted via the reducing end" or "the HAS is reacted via carbon atom C^* of the terminal reducing end" as used in the context of the present invention may relate to a process according to which the HAS is reacted predominantly via its (optionally selectively oxidised) reducing end.

[0121] This term "predominantly via its (optionally selectively oxidised) reducing end" relates to processes according to which statistically more than 50 %, preferably at least 55 %, more preferably at least 60 %, more preferably at least 65 %, more preferably at least 70 %, more preferably at least 75 %, more preferably at least 80 %, more preferably at least 85 %, more preferably at least 90 %, and still more preferably at least 95 % such as 95 %, 96 %, 97 %, 98 %, or 99 % of the HAS molecules employed for a given reaction are reacted via at least one (optionally selectively oxidised) reducing end per HAS molecule, wherein a given HAS molecule which is reacted via at least one (optionally selectively oxidised) reducing end can be reacted in the same given reaction via at least one further suitable functional group which is comprised in said polymer molecule and which is not a reducing end. If one or more HAS molecule(s) is (are) reacted via at least one (optionally selectively oxidised) reducing end and simultaneously via at least one further suitable functional group which is comprised in this (these) HAS molecule(s) and which is not a (optionally selectively oxidised) reducing end, statistically preferably more than 50 %, preferably at least 55 %, more preferably at least 60 %, more preferably at least 65 %, more preferably at least 70 %, more preferably at least 75 %, more preferably at least 80 %, more preferably at least 85 %, more preferably at least 90 %, and still more preferably at least 95 % such as 95 %, 96 %, 97 %, 98 %, or 99 % of all reacted functional groups of these HAS molecules, said functional groups including the (optionally selectively oxidised) reducing ends, are (selectively oxidised) reducing ends.

[0122] The term "reducing end" as used in the context of the present invention relates to the terminal aldehyde group of a HAS molecule which may be present as aldehyde group and/or as corresponding hemiacetal form and/or as acetal group, the acetal group having the following structure
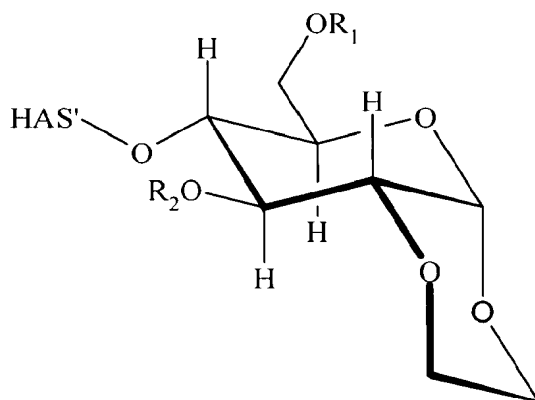
which can be present if residue -OR$_3$ according to formula (I) above is -O-CH$_2$-CH$_2$-OH.

[0123] In case the reducing end is oxidised, the oxidised reducing end is in the form of a carboxy group and/or of the corresponding lactone.

Oxidised reducing end

[0124] Therefore, according to a first embodiment of the present invention, the crosslinking compound is reacted via amino group with the oxidised C* atom of the terminal reducing end of HAS, preferably HES.

[0125] Although the oxidation may be carried out according to all suitable method or methods resulting in the oxidised reducing end of hydroxyalkyl starch, it is preferably carried out using an alkaline iodine solution as described, e.g., in Sommermeyer et al., US 6,083,909, column 5, lines 63-67, and column 7, lines 25-39; column 8, line 53 to column 9, line 20, the respective content being incorporated into the present invention by reference.

[0126] Selectively oxidising the HAS, preferably the HES leads to HAS, preferably HES being a lactone
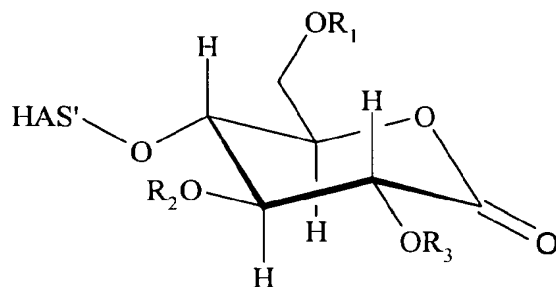
and/or a carboxylic acid
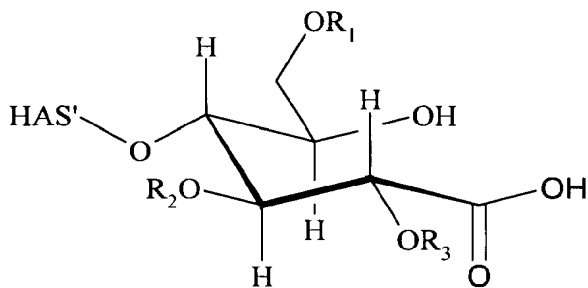
or a suitable salt of the carboxylic acid such as alkali metal salt, preferably as sodium and/or potassium salt, and HAS' preferably being HES'.

[0127] According to a first alternative of the present invention, this form of the HAS, preferably HES, is reacted as such with the amino group M of the crosslinking compound.

[0128] According to a second alternative of the present invention, the HAS, preferably HES, selectively oxidised at its reducing end, is first reacted with a suitable compound to give the HAS, preferably HES, comprising a reactive carboxy group.

[0129] Introducing the reactive carboxy group into the HAS which is selectively oxidised at its reducing end may carried out by all conceivable methods and all suitable compounds.

[0130] According to a specific method of the present invention, the HAS which is selectively oxidised at its reducing end is reacted at the oxidised reducing end with at least one alcohol, preferably with at least one acidic alcohol such as acidic alcohols having a pK_A value in the range of from 6 to 12 or of from 7 to 11 at 25 °C. The molecular weight of the acidic alcohol may be in the range of from 80 to 500 g/mol, such as of from 90 to 300 g/mol or of from 100 to 200 g/mol.

[0131] Suitable acidic alcohols are all alcohols $H-O-R_A$ having an acidic proton and are capable of being with reacted with the oxidised HAS to give the respective reactive HAS ester, preferably according to the formula
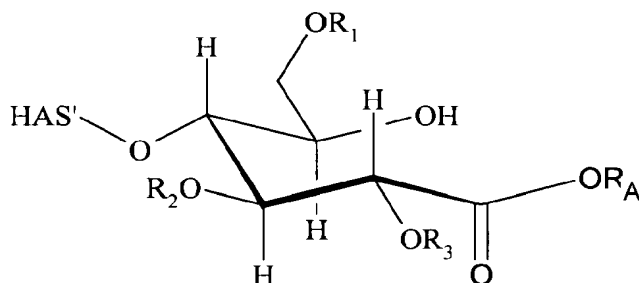
still more preferably according to formula
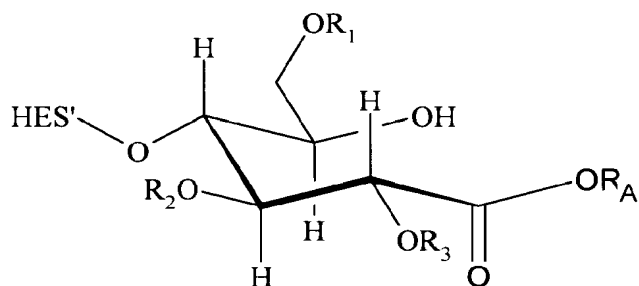
[0132] Preferred alcohols are N-hydroxy succinimides such as N-hydroxy succinimde or Sulfo-N-hydroxy succinimide, suitably substituted phenols such as p-nitrophenol, o,p-dinitrophenol, o,o'-dinitrophenol, trichlorophenol such as 2,4,6-trichlorophenol or 2,4,5-trichlorophenol, trifluorophenol such as 2,4,6-trifluorophenol or 2,4,5-trifluorophenol, pentachlorophenol, pentafluorophenol, or hydroxyazoles such as hydroxy benzotriazole. Especially preferred are N-hydroxy succinimides, with N-hydroxysuccinimide and Sulfo-N-hydroxysuccinimide being especially preferred. All alcohols may be employed alone or as suitable combination of two or more thereof. In the context of the present invention, it is also possible to employ a compound which releases the respective alcohol, e.g. by adding diesters of carbonic acid.

[0133] Therefore, the present invention also relates to a method as described above, wherein the HAS which is selectively oxidised at its reducing end is activated by reacting the oxidised HAS with an acidic alcohol, preferably with N-hydroxy succinimide and/or Sulfo-N-hydroxy succinimide.

[0134] According to a preferred embodiment of the present invention, the HAS which is selectively oxidised at its reducing end is reacted at the oxidised reducing end with at least one carbonic diester $R_B-O-(C=O)-O-R_C$, wherein R_B and R_C may be the same or different. Preferably, this method gives reactive HAS according to the formula

$$\text{HAS}' \diagdown \underset{\substack{\\ O}}{\overset{\substack{H \\ |}}{\diagdown}} \quad \overset{OR_1}{\underset{R_2O \diagdown \underset{H}{\overset{|}{\diagup}}}{\diagup}} \quad \overset{\substack{H \\ |}}{\underset{\substack{| \\ OR_3}}{\diagdown}} OH \quad OR_{B/C}$$

wherein HAS' is preferably HES'.

[0135] As suitable carbonic diester compounds, compounds may be employed whose alcohol components are independently N-hydroxy succinimides such as N-hydroxy succinimde or sulfo-N-hydroxy succinimide, suitably substituted phenols such as p-nitrophenol, o,p-dinitrophenol, o,o'-dinitrophenol, trichlorophenol such as 2,4,6-trichlorophenol or 2,4,5-trichlorophenol, trifluorophenol such as 2,4,6-trifluorophenol or 2,4,5-trifluorophenol, pentachlorophenol, pentafluorophenol, or hydroxyazoles such as hydroxy benzotriazole. Especially preferred are N,N'-disuccinimidyl carbonate and sulfo-N,N'-disuccinimidyl carbonate, with N,N'-disuccinimidyl carbonate being especially preferred.

[0136] Therefore, the present invention also relates a method as described above, wherein the HAS which is selectively oxidised at its reducing end is activated by reacting the oxidised HAS with N,N'-disuccinimidyl carbonate.

[0137] The acidic alcohol is reacted with the oxidised HAS or the salt of the oxidised HAS at a molar ratio of acidic alcohol : HAS preferably of from 5:1 to 50:1, more preferably of from 8:1 to 20:1, at a preferred reaction temperature of from 2 to 40 °C, more preferably of from 10 to 30 °C and especially preferably of from 15 to 25 °C. The reaction time is preferably in the range of from 1 to 10 h, more preferably of from 2 to 5 h, more preferably of from 2 to 4 h and particularly of from 2 to 3 h.

[0138] The carbonic diester compound is reacted with the oxidised HAS or the salt of the oxidised HAS at a molar ratio of diester compound : HAS generally of from 1:1 to 3:1, such as of from 1:1 to 1.5:1. The reaction time is generally in the range of from 0.1 to 12 h, like of from 0.2 to 6 h, or of from 0.5 to 2 h or of from 0.75 to 1.25 h.

[0139] According to a preferred embodiment of the present invention, reacting the oxidised HAS with acidic alcohol and/or carbonic diester is carried out in at least one aprotic solvent, such as in an anhydrous aprotic solvent having a water content of not more than 0.5 percent by weight, preferably of not more than 0.1 percent by weight. Suitable solvents are, among others, dimethyl sulfoxide (DMSO), N-methyl pyrrolidone, dimethyl acetamide (DMA), dimethyl formamide (DMF) and mixtures of two or more thereof. The reaction temperatures are preferably in the range of from 2 to 40 °C, more preferably of from 10 to 30 °C.

[0140] For reacting the oxidised HAS with the at least one acidic alcohol, at least one additional activating agent is employed.

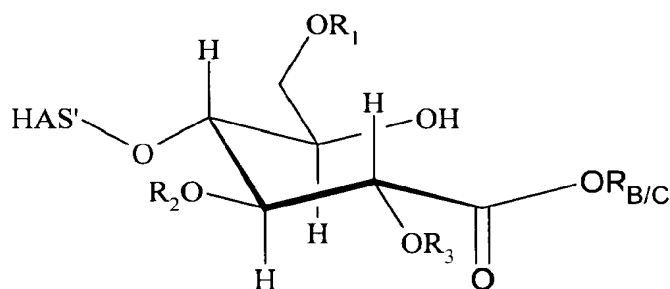**[0141]** Suitable activating agents are, among others, carbonyldiimidazole, carbodiimides such as diisopropyl carbodiimde (DIC), dicyclohexyl carbodiimides (DCC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), with dicyclohexyl carbodiimides (DCC) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) being especially preferred.

[0142] Therefore, the present invention also relates to the method as described above, where the HAS which is oxidised at its reducing end, is reacted with an acidic alcohol in the presence of an additional activating agent to give the reactive HAS ester.

[0143] According to one embodiment of the present invention, the reaction of the oxidised HAS with carbonic diester and/or acidic alcohol is carried out at a low base activity which may be determined by adding the reaction mixture to water with a volume ratio of water to reaction mixture of 10:1. Prior to the addition, the water which comprises essentially no buffer, has a pH value of 7 at 25 °C. After the addition of the reaction mixture and by measuring the pH value, the base activity of the reaction mixture is obtained, having a value of preferably not more than 9.0, more preferably of nor more than 8.0 and especially preferably of not more than 7.5.

[0144] According to another embodiment of the present invention, the oxidised HAS is reacted with N-hydroxy succinimide in dry DMA in the absence of water with EDC to selectively give the polymer N-hydroxy succinimide ester according to the formula
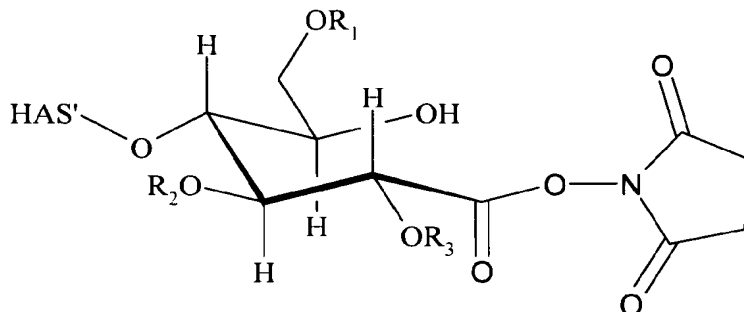
more preferably with HAS' being HES'.

[0145] This reaction does not give by-products resulting from reactions of EDC with OH groups of HES, and the rearrangement reaction of the O-acyl isourea formed by EDC and the oxidised HAS to the respective N-acyl urea is surprisingly suppressed.

[0146] According to another preferred embodiment of the present invention, the oxidised HAS is reacted with N,N'-disuccinimidyl carbonate in dry DMF in the absence of water and in the absence of an activating agent to selectively give the HAS-N-hydroxy succinimide ester according to the formula
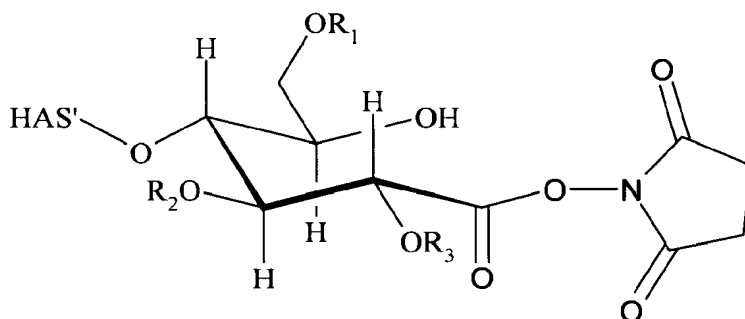
more preferably with HAS' being HES'.

[0147] According to another embodiment of the present invention, the HAS which is selectively oxidised at its reducing end is reacted at the oxidised reducing end with an azolide such as carbonyldiimidazole or carbonyl dibenzimidazole to give a polymer having a reactive carboxy group. In the case of carbonyldiimidazole, a reactive imidazolide HAS derivative according to formula
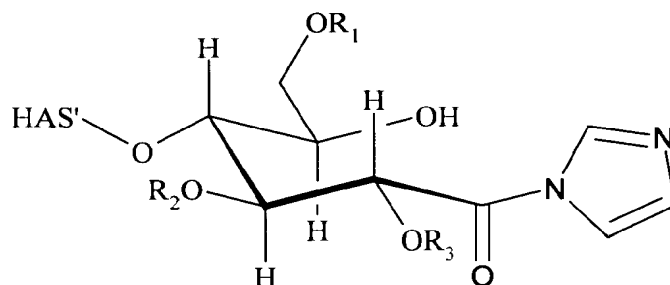
results, wherein HAS' is preferably HES'.

[0148] The reactive HAS derivative comprising at least one reactive carboxy group, preferably resulting from the reaction of the HAS with the acidic alcohol, the carbonate and/or the azolide, as described above, is then further reacted

with the amino group M of the crosslinking compound M.

[0149] Reaction of the HAS via the oxidised reducing end, optionally further activated as described above, with amino group M can be carried out according to all suitable methods. Preferably, the amino group M is a primary amino group H_2N- or a secondary amino group.

[0150] Generally, preferably polar aprotic solvents are used which may also contain a certain amount of water, such as up to 10 wt.-%. Preferred aprotic solvents are, among others, DMSO or DMF.

[0151] An example of a preferred reaction temperature range is from 0 to 80 °C, more preferably from 0 to 70 °C, more preferably from 0 to 60 °C, more preferably from 0 to 50 °C and even more preferably from 0 to 40 °C.

[0152] If crosslinking compounds are used for reaction with HAS having the reducing end in oxidised form which, according to a preferred embodiment, have H_2N- as amino group M, a HAS derivative is obtained by step (i) of the present invention wherein the HAS and the crosslinking compound employed as starting materials are linked via an amid bond, wherein the obtained HAS derivative further contains the acetal or keto group A.

[0153] Therefore, the present invention also relates to the method as described above, wherein in (i), HAS is reacted via its oxidised reducing end with the amino group M of the crosslinking compound, M being H_2N-, and wherein the reaction is carried out at a temperature in the range of from 0 to 80 °C, and wherein X is -(C=O)-NH-.

[0154] Accordingly, the present invention also relates to the HAS derivative, obtainable or obtained by the method as described above.

[0155] Moreover, the present invention also relates to the HAS derivative as such, having the following structure
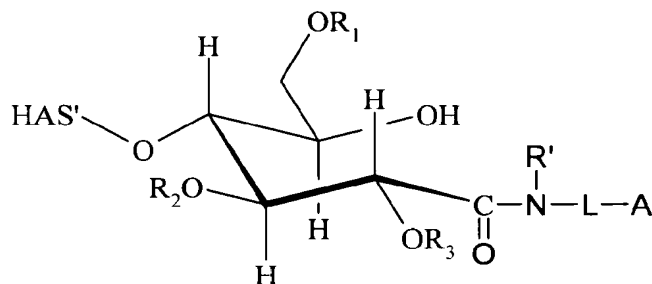
wherein R' is H if the amino group M of the crosslinking compound is a primary amino group, and wherein R' is a chemical moiety other than H if the amino group M of the crosslinking compound is a secondary amino group. The precise chemical nature of R' is dependent on the crosslinking compound, and thus, reference is made to the discussion of the generally possible and preferably employed crosslinking compounds hereinabove.

[0156] In accordance with above-described preferred crosslinking compounds employed for the present invention, the following HAS derivatives may be mentioned as preferred embodiments by way of example, wherein in each case, HAS is - according to preferred embodiments of the present invention - HES:

or

or

or

or

or

or
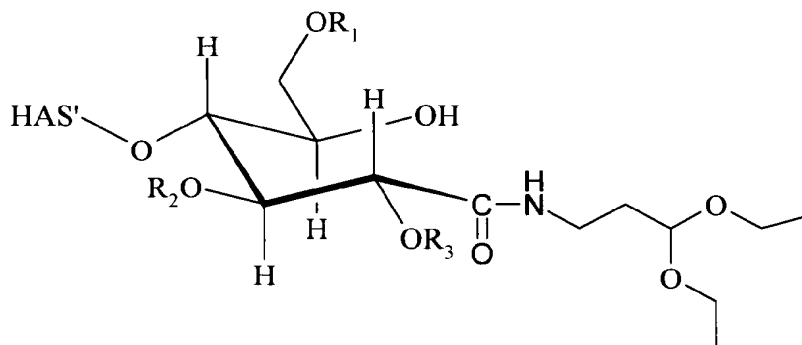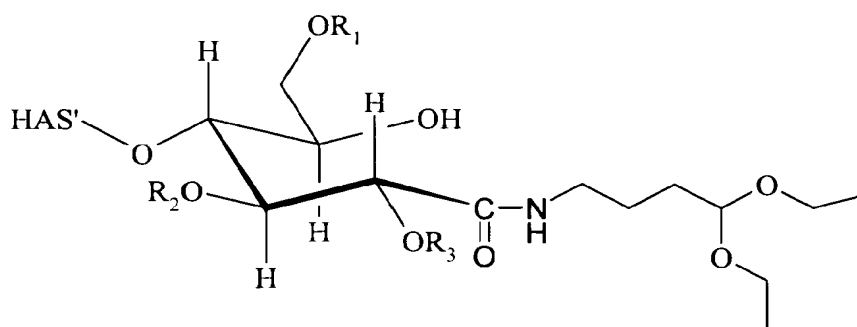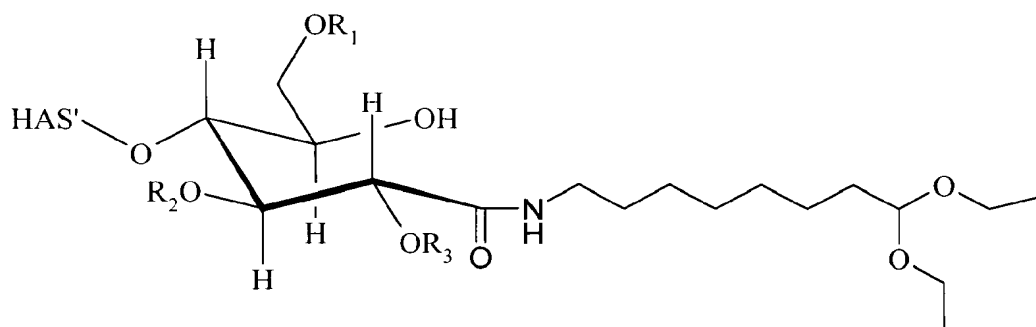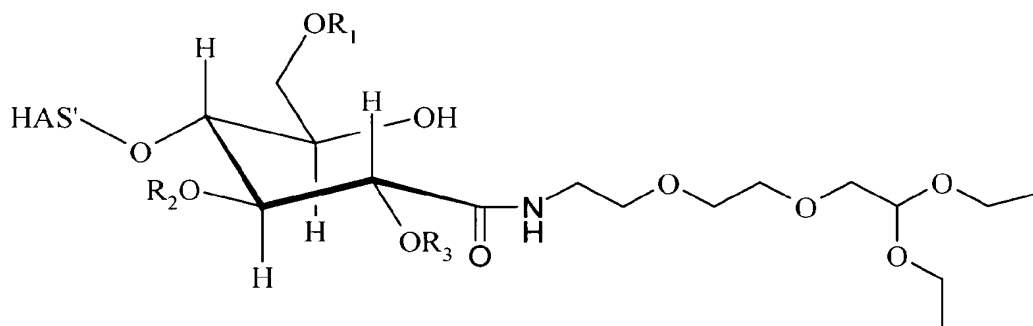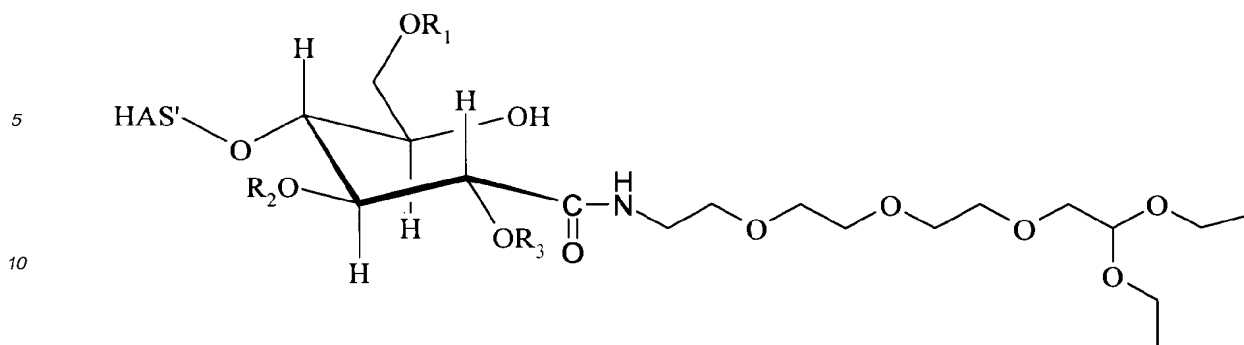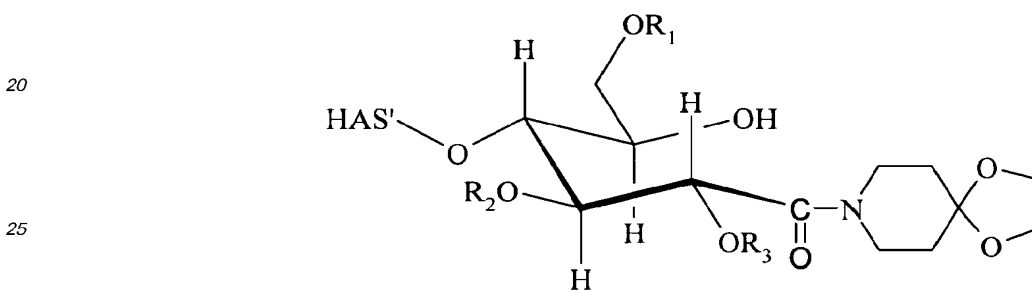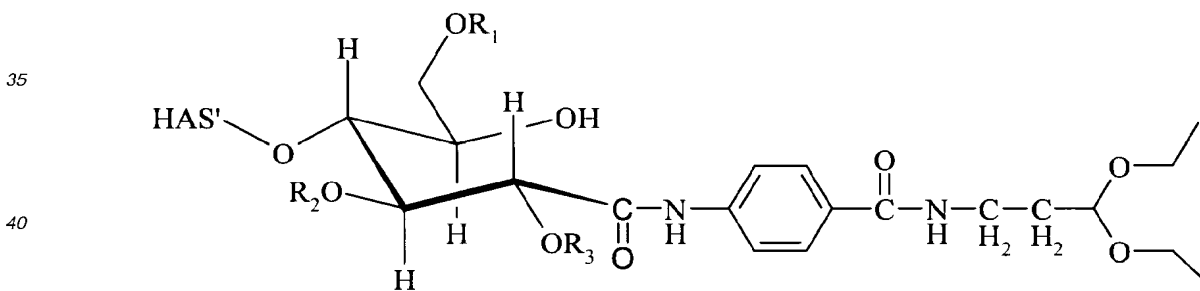
or
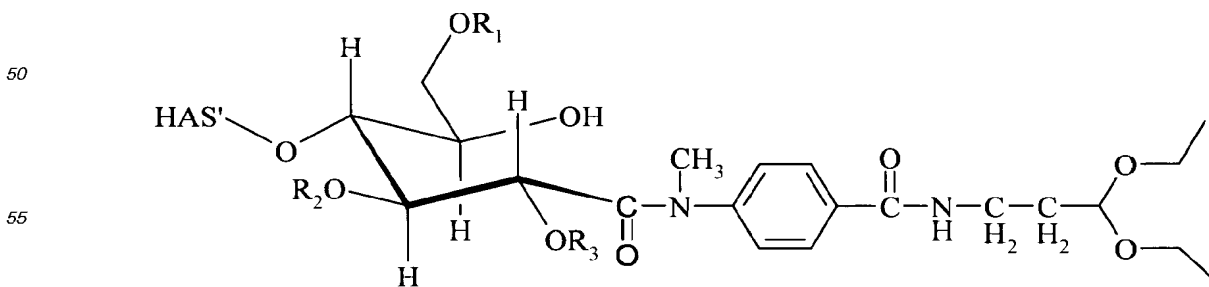
[0157] According to an especially preferred embodiment, the present invention relates to a HES derivative having the following structure:

wherein, even more preferably, HES has a mean molecular weight from about 1 to about 500 kDa, more preferably from about 2 to 400 kDa, more preferably from about 5 to about 300 kDa, more preferably from about 10 to about 200 kDa, in particular from about 50 to about 150 kDa, a molar substitution of 0.1 to 3, preferably 0.4 to 1.3, such as 0.4, 0.5, 0.6, 0.7 0.8, 0.9, 1.0, 1.1, 1.2, or 1.3, and a ratio of $C_2 : C_6$ substitution of preferably in the range of from 2 to 20, more preferably in the range of from 2 to 15 and even more preferably in the range of from 3 to 12.

Non-oxidised reducing end

[0158] According to a second and preferred embodiment of the present invention, the crosslinking compound is reacted via amino group with the non-oxidised C* atom of the terminal reducing end of HAS, preferably HES, i.e. the terminal aldehyde group of a HAS molecule may be present as aldehyde group and/or as corresponding hemiacetal form.

[0159] Reaction of the HAS via the non-oxidised reducing end, with amino group M can be carried out according to all suitable methods. Preferably, the amino group M is H_2N-, a suitable secondary amino group HNR'- such as, e.g., H_3C-NH-, H_2N-O-, or a suitable secondary hydroxyamino group HNR'-O- such as, e.g., H_3C-NH-O-, or H_2N-NH-(C=O)-.

[0160] Preferably, the amino group M is H_2N-, H_2N-O- or H_2N-NH-(C=O)-, even more preferably H_2N- or H_2N-O-, and in particular H_2N-.

[0161] According to a preferred embodiment of the present invention, this reaction is carried out in an aqueous system. The term "aqueous system" as used in this context of the present invention refers to a solvent or a mixture of solvents comprising water in the range of from at least 10 % per weight, preferably at least 50 % per weight, more preferably at least 80 % per weight, even more preferably at least 90 % per weight or up to 100 % per weight, based on the weight of the solvents involved. As additional solvents, solvents such as DMSO, DMF, ethanol or methanol may be mentioned.

[0162] According to a preferred embodiment, if HAS is reacted with the crosslinking compound in an aqueous medium and the amino group M of the crosslinking compound is a hydroxylamine or a hydrazide, the temperature of the reaction is preferably in the range of from 5 to 45 °C, more preferably in the range of from 10 to 30 °C and especially preferably in the range of from 15 to 25 °C.

[0163] According to another preferred embodiment, if HAS is reacted with the crosslinking compound in an aqueous medium and the amino group M of the crosslinking compound is a group H_2N- or $R'HN$-, the reaction being a reductive amination, the temperature is preferably in the range of up to 100 °C, more preferably in the range of from 10 to 90 °C, more preferably in the range of from 20 to 80 °C, more preferably in the range of from 30 to 70 °C and especially preferably in the range of from 40 to 60 °C.

[0164] During the course of the reaction the temperature may be varied, preferably in the above-given ranges, or held essentially constant.

[0165] The reaction time for the reaction of HAS with crosslinking compound M may be adapted to the specific needs and is generally in the range of from 1 h to 7 d. In case, e.g., amino group M is a hydroxylamine or a hydrazide, the reaction time is preferably in the range of from 1 h to 3 d, more preferably of from 2 h to 48 h, and especially preferably of from 3 to 24 h.

[0166] In case, e.g., the reaction of HAS with the crosslinking compound is a reductive amination, the reaction time is preferably in the range of from 1 h to 7 d, more preferably in the range of from 4 h to 6 d, more preferably in the range of from 8 h to 5 d and even more preferably in the range of from 16 h to 3 d.

[0167] The pH value for the reaction of HAS with the crosslinking compound may be adapted to the specific needs

such as the chemical nature of the reactants. In case, e.g., group M of the crosslinking compound is a hydroxylamine or a hydrazide, the pH value is preferably in the range of from 3 to 9, more preferably of from 4 to 8 and even more preferably of from 4.5 to 6.5.

[0168] In case, e.g., the reaction of HAS with the crosslinking compound is a reductive amination, the pH value is preferably in the range of from 3 to 9, more preferably in the range of from 3.5 to 8, and even more preferably in the range of from 4 to 6.

[0169] The suitable pH value of the reaction mixture may be adjusted, for each reaction step, by adding at least one suitable buffer. Among the preferred buffers, sodium acetate buffer, phosphate or borate buffers may be mentioned.

[0170] If crosslinking compounds are used for reaction with HAS having the reducing end in non-oxidised form which, according to a preferred embodiment, have H_2N- as amino group M, a HAS derivative is obtained by step (i) of the present invention wherein the HAS and the crosslinking compound employed as starting materials are linked via an imine bond, wherein the obtained HAS derivative further contains the acetal or keto group A. If the reaction is carried out under reductive amination conditions in the presence of a suitable reducing agent, a HAS derivative is obtained by step (i) of the present invention wherein the HAS and the crosslinking compound employed as starting materials are linked via an amine bond, wherein the obtained HAS derivative further contains the acetal or keto group A.

[0171] Therefore, the present invention also relates the method as described above, wherein in (i), HAS is reacted, preferably in an aqueous system, via its non-oxidised reducing end with the amino group M of the crosslinking compound, M being H_2N-, and wherein the reaction is carried out at a temperature in the range of from 20 to 80 °C at a pH in the range of from 4 to 7, X being -CH=N-.

[0172] Further, the present invention also relates the above-described method, wherein in (i), the reaction is carried out in the presence of a reducing agent, such as sodium borohydride, sodium cyanoborohydride, organic borane complex compounds such as a 4-(dimethylamin)pyridine borane complex, N-ethyldiisopropylamine borane complex, N-ethylmorpholine borane complex, N-methylmorpholine borane complex, N-phenylmorpholine borane complex, lutidine borane complex, triethylamine borane complex, or trimethylamine borane complex, preferably $NaCNBH_3$, to obtain a HAS derivative, X being $-CH_2-NH-$.

[0173] The concentration of these reducing agents used for the reductive amination of the present invention is preferably in the range of from 0.01 to 2.0 mol/l, more preferably in the range of from 0.05 to 1.5 mol/l, and more preferably in the range of from 0.1 to 1.0 mol/l, relating, in each case, to the volume of the reaction solution.

[0174] According to above-described preferred embodiment wherein M is H_2N- and reaction of the crosslinking compound with HAS is carried out under reductive amination conditions, the molar ratio of crosslinking compound : HAS is preferably in the range of from 1:1 to 100:1, more preferably from 2:1 to 80:1, more preferably from 3:1 to 70:1, more preferably from 4:1 to 60:1, and more preferably from 5:1 to 50:1.

[0175] According to above-described preferred embodiment wherein M is H_2N- and reaction of the crosslinking compound with HAS is carried out under reductive amination conditions, the concentration of HAS, preferably HES, in the aqueous system is preferably in the range of from 1 to 50 wt.-%, more preferably from 3 to 45 wt.-%, and more preferably from 5 to 40 wt.-%, relating, in each case, to the weight of the reaction solution.

[0176] If crosslinking compounds are used for reaction with HAS having the reducing end in non-oxidised form which, according to a preferred embodiment, have H_2N-O- or $H_2N-NH-(C=O)-$ as amino group M, a HAS derivative is obtained by step (i) of the present invention wherein the HAS and the crosslinking compound employed as starting materials are linked via an -CH=N-O- bond or -CH=N-NH-(C=O)- bond, wherein the obtained HAS derivative further contains the acetal or keto group A. If the reaction is carried out under reducing conditions in the presence of a suitable reducing agent, a HAS derivative is obtained by step (i) of the present invention wherein the HAS and the crosslinking compound employed as starting materials are linked via an $-CH_2-NH-O-$ bond or $-CH_2-NH-NH-(C=O)-$ bond, wherein the obtained HAS derivative further contains the acetal or keto group A.

[0177] Therefore, the present invention also relates the method as described above, wherein in (i), HAS is reacted, preferably in an aqueous system, via its non-oxidised reducing end with the amino group M of the crosslinking compound, M being H_2N-O- or $H_2N-NH-(C=O)-$, and wherein the reaction is carried out at a temperature in the range of from 5 to 80 °C at a pH in the range of from 4.5 to 6.5, X being -CH=N-O- or -CH=N-NH-(C=O)-.

[0178] Further, the present invention also relates the above-described method, wherein in (i), the reaction is carried out in the presence of a reducing agent, such as sodium borohydride, sodium cyanoborohydride, organic borane complex compounds such as a 4-(dimethylamin)pyridine borane complex, N-ethyldiisopropylamine borane complex, N-ethylmorpholine borane complex, N-methylmorpholine borane complex, N-phenylmorpholine borane complex, lutidine borane complex, triethylamine borane complex, or trimethylamine borane complex, preferably $NaCNBH_3$, to obtain a HAS derivative, X being $-CH_2-NH-O-$ or $-CH_2-NH-NH-(C=O)-$.

[0179] The concentration of these reducing agents used for this reaction of the present invention is preferably in the range of from 0.001 to 2.0 mol/l, more preferably in the range of from 0.01 to 1.0 mol/l, and more preferably in the range of from 0.1 to 0.8 mol/l, relating, in each case, to the volume of the reaction solution.

[0180] According to above-described preferred embodiment wherein M is H_2N-O- or $H_2N-NH-(C=O)-$, and the reaction

of the crosslinking compound with HAS is carried out under reducing conditions, the molar ratio of crosslinking compound : HAS is preferably in the range of from 1:1 to 100:1, more preferably from 2:1 to 80:1, more preferably from 3:1 to 70:1, more preferably from 4:1 to 60:1, and more preferably from 5:1 to 50:1.

[0181] According to above-described preferred embodiment wherein M is H_2N- and reaction of the crosslinking compound with HAS is carried out under reductive amination conditions, the concentration of HAS, preferably HES, in the aqueous system is preferably in the range of from 1 to 50 wt.-%, more preferably from 3 to 45 wt.-%, and more preferably from 5 to 40 wt.-%, relating, in each case, to the weight of the reaction solution.

[0182] Accordingly, the present invention also relates to the HAS derivative, obtainable or obtained by the method(s) as described above.

[0183] Moreover, the present invention also relates to the HAS derivative as such, having the following structure
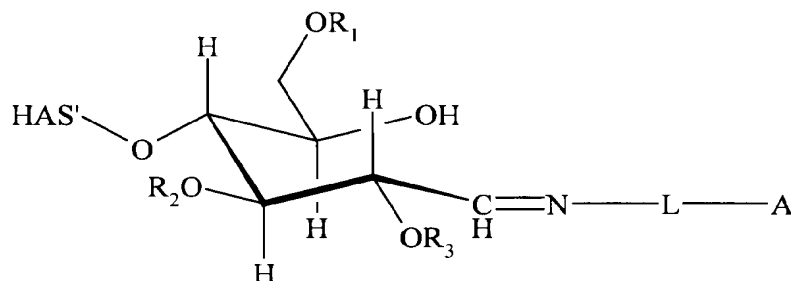
wherein, depending on the reaction conditions and/or the specific chemical nature of the crosslinking compound, the C-N double bond may be present in E or Z conformation where also a mixture of both forms may be present having a certain equilibrium distribution;

or, as far as the corresponding ring structure is concerned which for the purposes of the present invention shall be regarded as identical to the open structure above,
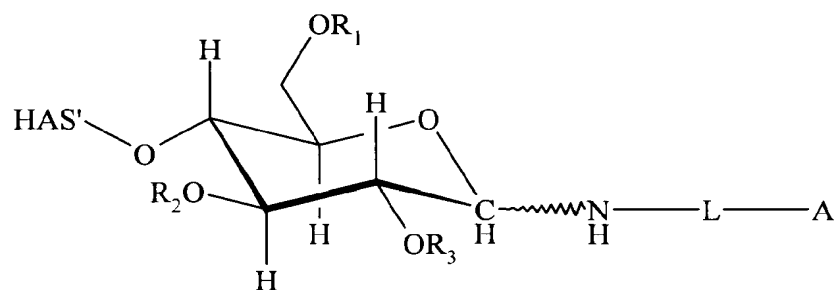
wherein depending on the reaction conditions and/or the specific chemical nature of crosslinking compound, these HAS derivatives may be present with the N atom in equatorial or axial position where also a mixture of both forms may be present having a certain equilibrium distribution;

or

or
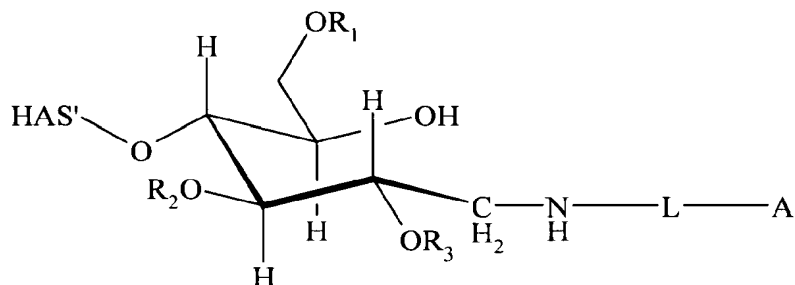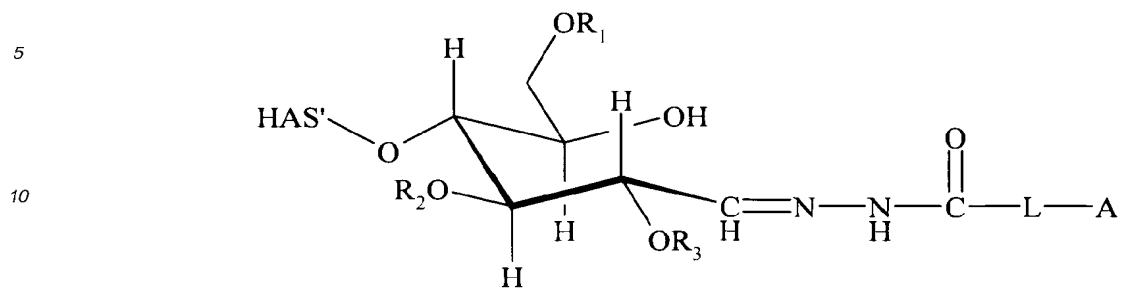
or the corresponding ring structure

or

or

or the corresponding ring structure

or
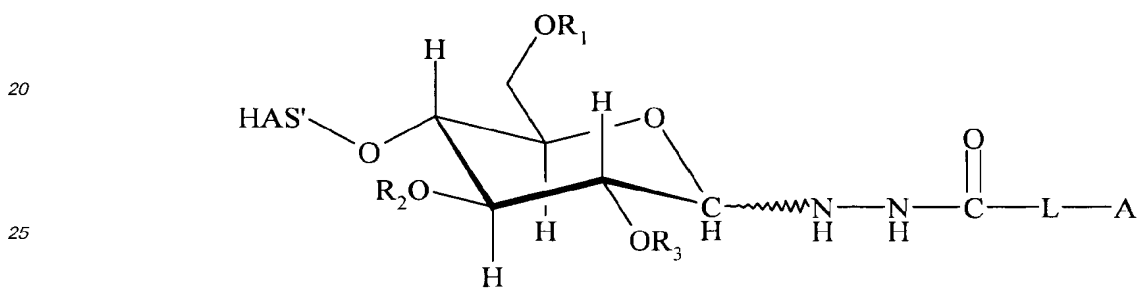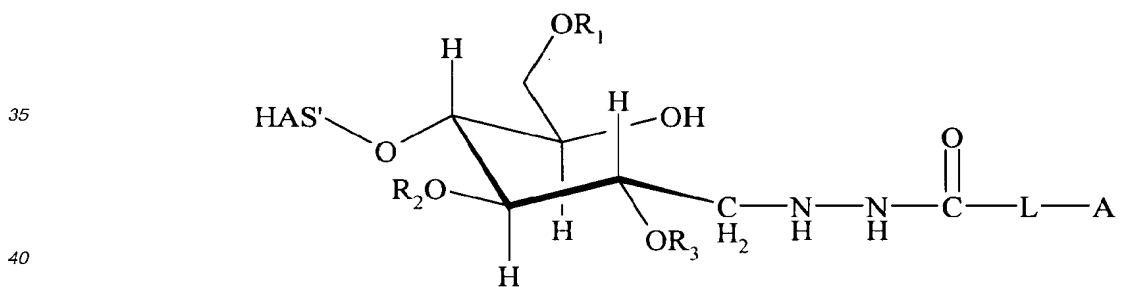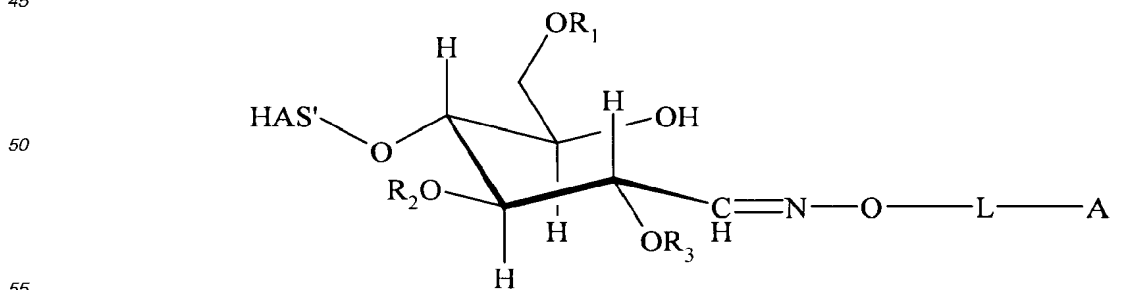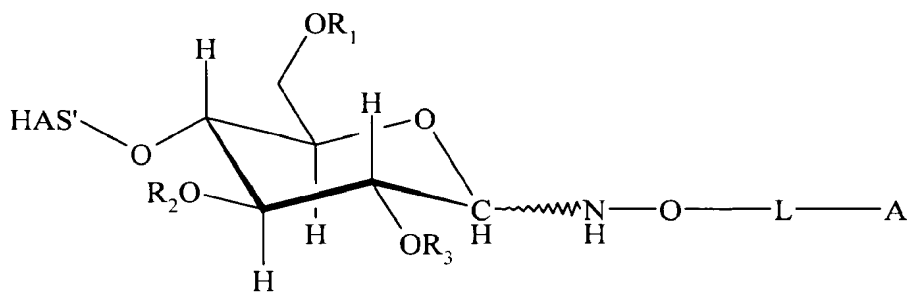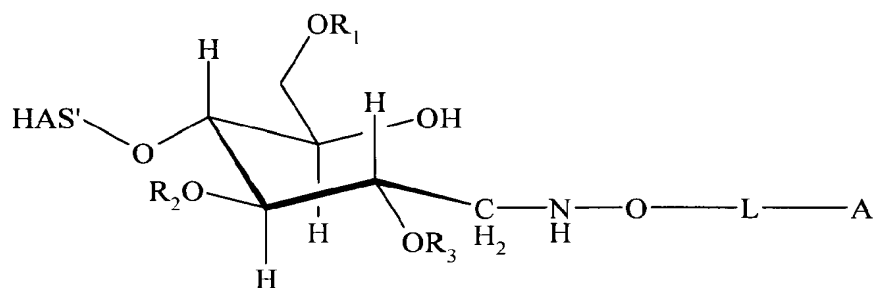
[0184]   In accordance with above-described preferred crosslinking compounds, the following HAS derivatives may be mentioned as preferred embodiments by way of example, wherein in each case, HAS is - according to preferred embodiments of the present invention - HES:

wherein the corresponding ring structure is included,
or

or

wherein the corresponding ring structure is included,
or

or
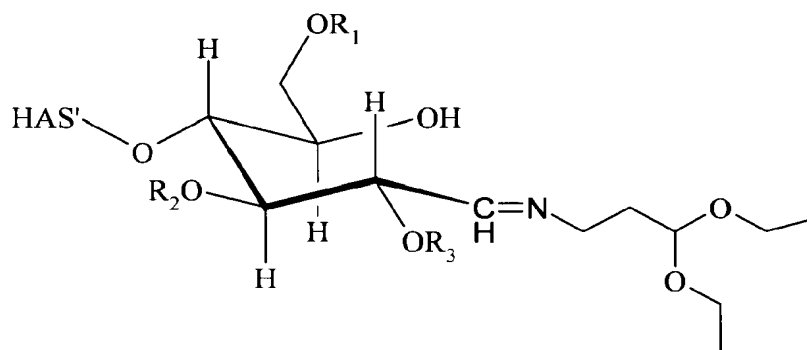
wherein the corresponding ring structure is included,
or

5
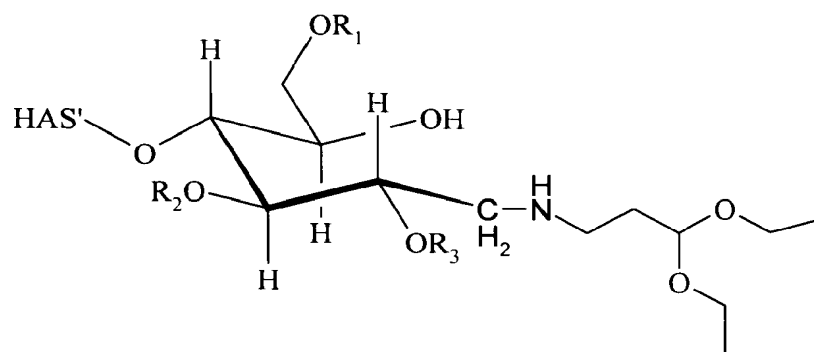
10

15

or

20
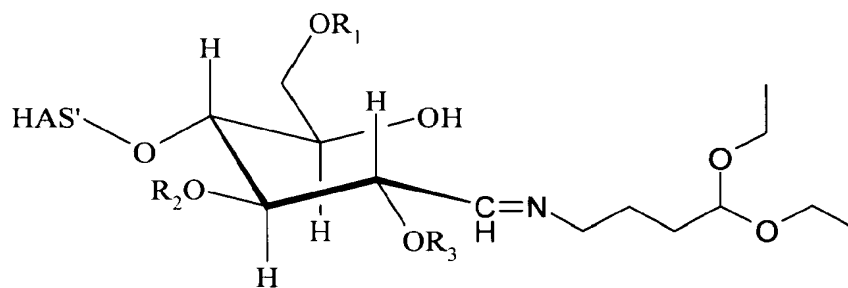
25

30
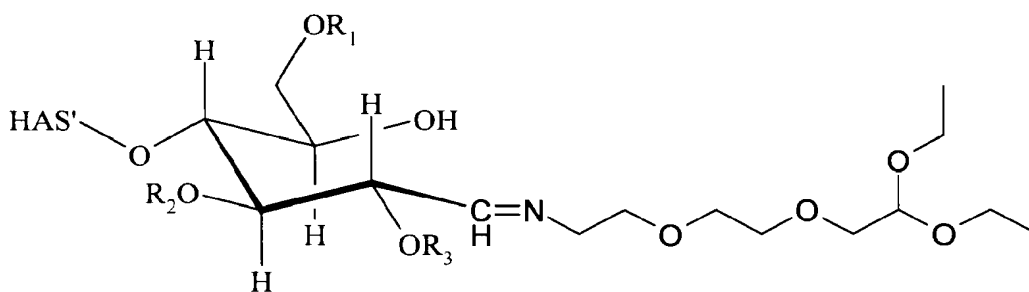
wherein the corresponding ring structure is included,
or

35

40

45

or

50
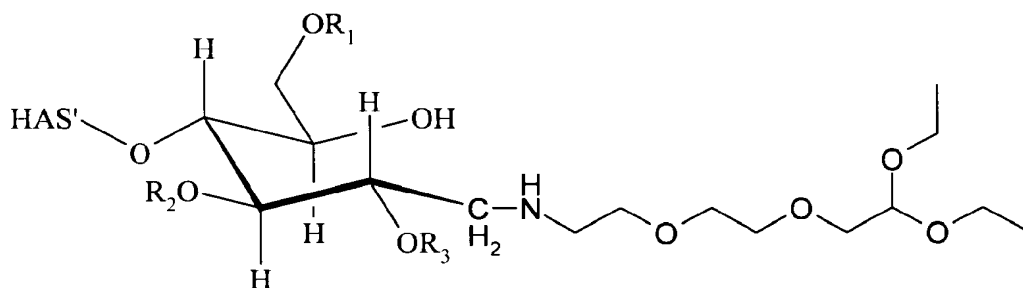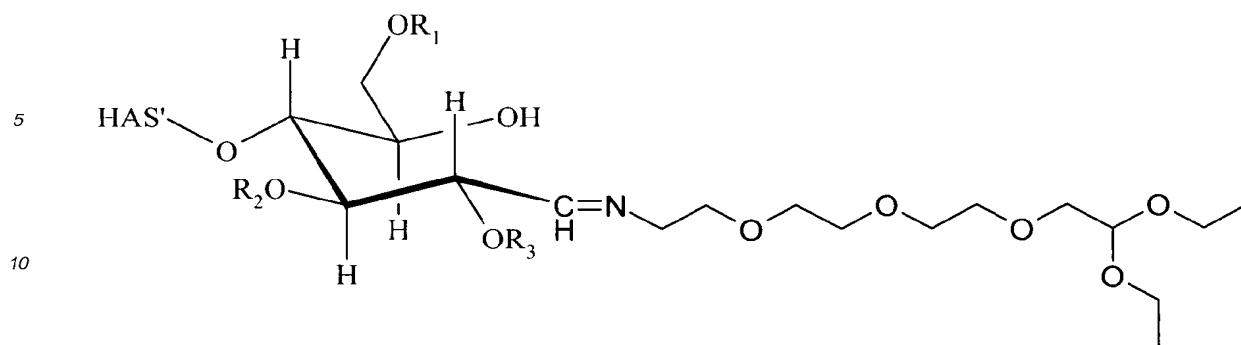
55

wherein the corresponding ring structure is included,
or

or

or
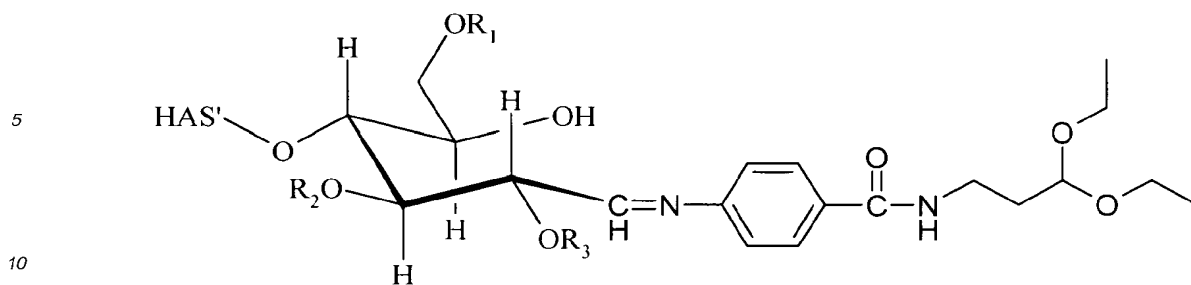
wherein the corresponding ring structure is included,
or

wherein the corresponding ring structure is included,
or

or

wherein the corresponding ring structure is included,
or

or

wherein the corresponding ring structure is included, or

[0185]   According to an especially preferred embodiment, the present invention relates to a HES derivative having the following structure:

the corresponding ring structure

and/or

wherein, even more preferably, HES has a mean molecular weight from about 1 to about 500 kDa, more preferably from about 2 to 400 kDa, more preferably from about 5 to about 300 kDa, more preferably from about 10 to about 200 kDa, in particular from about 50 to about 150 kDa, a molar substitution of 0.1 to 3, preferably 0.4 to 1.3, such as 0.4, 0.5, 0.6, 0.7 0.8, 0.9, 1.0, 1.1, 1.2, or 1.3, and a ratio of C_2 : C_6 substitution of preferably in the range of from 2 to 20, more preferably in the range of from 2 to 15 and even more preferably in the range of from 3 to 12.

[0186] Other conceivable embodiments with regard to HAS

[0187] For the sake of completeness, it shall be mentioned that, while not preferred according to the present invention, it might be conceivable that HAS is oxidised prior to the reaction with the crosslinking compound such that at least two aldehyde groups would be introduced into HAS according to the following formula
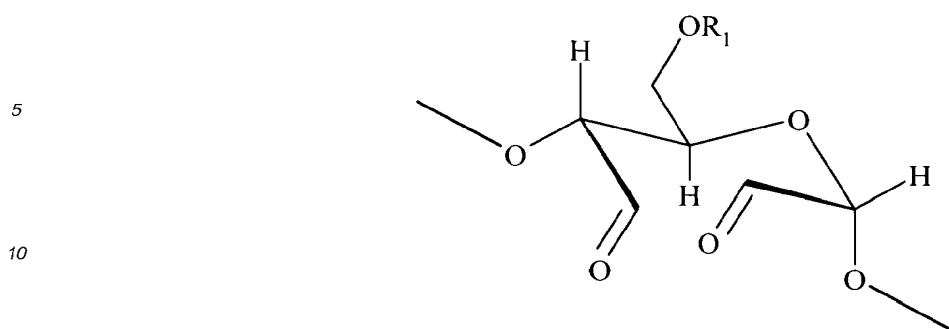
[0188] Generally, each oxidation agent or combination of oxidation agents might be employed capable of oxidising at least one saccharide ring of the polymer to give an opened saccharide ring having at least one, preferably at least two aldehyde groups. This reaction might be illustrated by the following reaction scheme showing a saccharide ring of HAS which is oxidised to give an opened ring having two aldehyde groups:
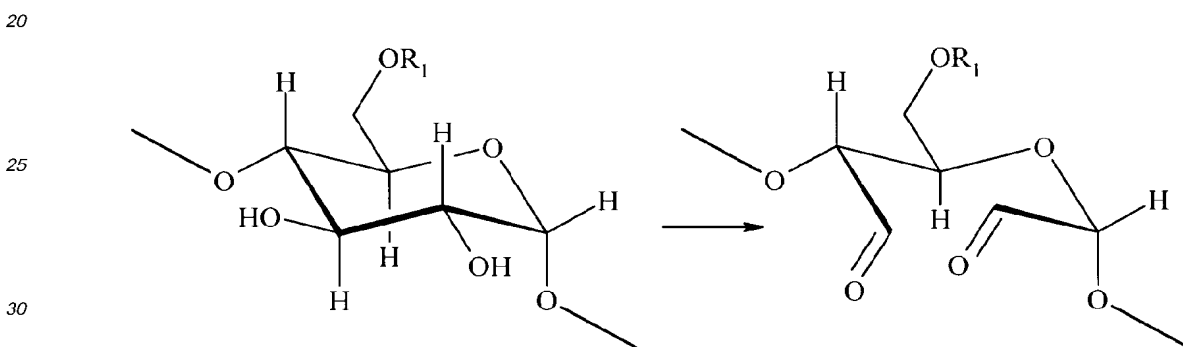
[0189] Suitable oxidising agents are, among others, periodates such as alkaline metal periodates or mixtures of two or more thereof, with sodium periodate and potassium periodate being preferred. It might be conceivable that these aldehyde groups could be reacted with the crosslinking compound M-L-A via amino group M.

Isolation and/or purification

[0190] Generally, it is conceivable that the HAS derivative from step (i) of the present invention is subsequently reacted as described hereinunder. According to a preferred embodiment, the HAS derivative from step (i) is suitably purified after the reaction step (i).

[0191] For the purification of the HAS derivative from step (i), the following possibilities A) to C) can be mentioned by way of example, wherein possibility A) is preferred:

A) Ultrafiltration using water or an aqueous buffer solution having a concentration preferably of from 0.1 to 100 mmol/l, more preferably from 1 to 50 mmol/l and more preferably from 5 to 20 mmol/l such as about 10 mmol/ml, a pH in the range of preferably from 2 to 10, more preferably from 4 to 10, more preferably from 6 to 10 and more preferably from 8 to 10 such as about 9; the number of exchange cycles preferably is from 10 to 50, more preferably from 10 to 40 and even more preferably from 10 to 30 such as about 20.

B) Dialysis using water or aqueous buffer solution having a concentration preferably of from 0.1 to 100 mmol/l, more preferably from 1 to 50 mmol/l and more preferably from 5 to 20 mmol/l such as about 10 mmol/ml, a pH in the preferred range of from 2 to 10, more preferably from 4 to 10, more preferably from 6 to 10 and more preferably from 7 to 9; wherein a solution is employed containing the HAS derivative in a preferred concentration of from 5 to 20 wt.-%; and wherein buffer or water is used in particular in an excess of about 100:1 1 to the HES derivative solution.

C) Precipitation with ethanol or isopropanol, centrifugation and re-dissolving in water to obtain a solution having a

preferred concentration of about 10 wt.-%, and subsequent ultrafiltration using water or an aqueous buffer solution having a concentration of preferably from 0.1 to 100 mmol/l, more preferably from 1 to 50 mmol/l and even more preferably from 5 to 20 mmol/l such as about 10 mmol/ml, a pH in the preferred range of from 2 to 10, more preferably from 4 to 10, more preferably from 6 to 10 and more preferably from 7 to 9; the number of exchange cycles is preferably from 10 to 40, more preferably from 10 to 30 and even more preferably from 10 to 20 such as 10.

[0192] After the preferred purification step, the HAS derivative is preferably obtained as a solid. According to further conceivable embodiments of the present invention, HAS derivative solutions or frozen HAS derivative solutions may be mentioned having preferred HAS derivative contents of from 2 to 40 wt.-%, wherein the pH of these solutions is preferably in a range of from 3 to 10 and the concentration of the buffer used is preferably in the range of from 0.1 to 1 mol/l.

[0193] Therefore, the present invention also relates to a method as described above, wherein, after (i), the HAS derivative obtained in (i) is purified using ultrafiltration using water or an aqueous buffer solution having a concentration of from 0.1 to 100 mmol/l, a pH in the range of from 2 to 10, the number of exchange cycles being from 10 to 50.

Reaction with biologically active agent BA

[0194] According to a further preferred embodiment, the present invention relates to a method wherein above-described HES derivative
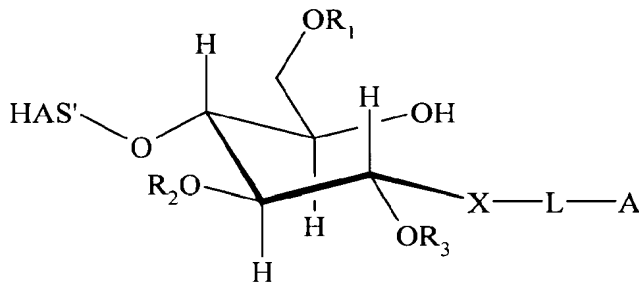
is further suitably reacted with a biologically active compound BA via acetal or ketal group A, which group A is preferably transformed to the corresponding aldehyde or keto group prior to the reaction with BA.

[0195] Most preferably, group A, preferably the corresponding aldehyde or keto group is reacted with an amino group, still more preferably with a primary amino group comprised in BA. For such cases and for the purposes of the present invention, BA is also represented as H_2N-BA' wherein BA' is the remainder of BA.

[0196] Therefore, the present invention also relates to the method as described above, further comprising

(ii) reacting the HAS derivative according to formula (III) via group A with an amino group of a biologically active agent H_2N-BA', via reductive amination, obtaining a HAS derivative according to formula (IV)

(IV)
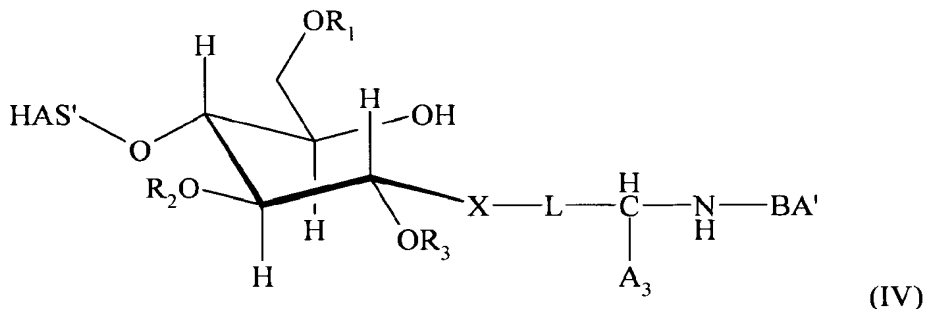
[0197] According to a first embodiment of the present invention, the HAS derivative obtained from (i) which has been preferably purified is suitably subjected to a transformation of group A to the corresponding aldehyde or keto group wherein the resulting HAS derivative is subjected to a suitable purification and/or isolation step prior to the reaction with BA.

[0198] The transformation to the aldehyde or keto group is preferably performed by an acid-catalyzed hydrolysis

reaction. The reaction is preferably carried out at a temperature of from 0 to 100 °C, more preferably from 10 to 80 °C and more preferably from 20 and 60 °C, at a pH which is preferably in the range of from 1 to 6, more preferably from 1 to 5, more preferably from 1 to 4, more preferably from 1 to 3 and even more preferably from 1 to less than 3. Purification and buffer-exchange of the hydrolysis reaction product can be achieved by methods well-known to those skilled in the art, e.g. by dialysis or ultrafiltration. The transformed material can be recovered from the solution as a solid e.g. by freeze-drying.

[0199] According to a second embodiment of the present invention, the HAS derivative obtained from (i) which has been preferably purified is suitably subjected to a transformation of group A to the corresponding aldehyde or keto group wherein the resulting HAS derivative is directly reacted with BA, i.e. without a separate suitable purification and/or isolation step of the HAS derivative comprising the aldehyde or keto group. The transformation to the aldehyde or keto group is preferably performed by an acid-catalyzed hydrolysis reaction. The reaction is preferably carried out at a temperature of from 0 to 100 °C, more preferably from 10 to 80 °C and more preferably from 20 and 60 °C, at a pH which is preferably in the range of from 1 to 6, more preferably from 1 to 5, more preferably from 1 to 4, more preferably from 1 to 3 and even more preferably from 1 to less than 3. The hydrolysis reaction product can be combined with the BA in a buffered solution either directly or after having adjusted the pH to a value compatible with the reaction with the BA.

[0200] Therefore, the present invention also relates to a method as described above wherein prior to (ii), group A of the HAS derivative according to formula (III) is transformed to the corresponding aldehyde or keto group.

[0201] According to a third conceivable embodiment of the present invention, the HAS derivative obtained from (i) which has been preferably purified is directly reacted with BA, i.e. reacted with BA under reaction conditions allowing for the *in situ* transformation of group A to the corresponding aldehyde or keto group without a separate suitable purification and/or isolation step and without a separate step for the transformation of group A to the corresponding aldehyde or keto group. The transformation to the aldehyde or keto group is preferably performed by an acid-catalyzed hydrolysis reaction. The reaction is preferably carried out at a temperature of from 0 to 100 °C, more preferably from 10 to 80 °C and more preferably from 20 and 60 °C, at a pH which is preferably in the range of from 1 to 6, more preferably from 1 to 5, more preferably from 1 to 4, more preferably from 1 to 3 and even more preferably from 1 to less than 3. The hydrolysis reaction product can be combined with the BA in a buffered solution either directly or after having adjusted the pH to a value compatible with the reaction with the BA.

[0202] Which method according to the above-mentioned three embodiments is carried out depends, for example, on the specific nature of the biologically active substance BA employed. If, e.g., a protein such as EPO, G-CSF or IFN alpha is employed as BA, above-identified first or second embodiment is generally suitable.

[0203] The reaction in step (ii) is preferably carried out in an aqueous system. The term "aqueous system" as used in this context of the present invention refers to a solvent or a mixture of solvents comprising water in the range of from at least 10 % per weight, preferably at least 50 % per weight, more preferably at least 80 % per weight, even more preferably at least 90 % per weight or up to 100 % per weight, based on the weight of the solvents involved. As additional solvents, solvents such as DMSO, DMF, ethanol or methanol may be mentioned.

[0204] While it is conceivable to carry out the reaction in step (i) under conditions to obtain the non-reduced form of the HAS derivative according to formula (IV), i.e.

$$(IV)$$
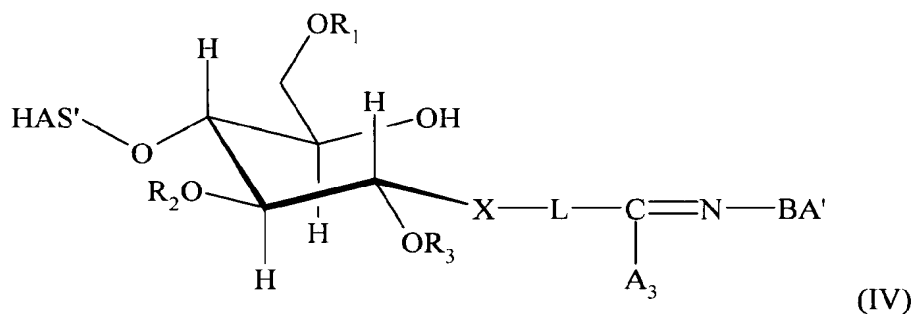
it is particularly preferred to carry out the reaction according to step (ii) under reductive amination conditions in the presence of at least one suitable reducing agent. In particular, under these conditions the group -CH=N- obtained through the reaction of the aldehyde or keto group resulting from group A of the HAS derivative and the H_2N-group of BA is reduced to $-CH_2-NH-$.

[0205] By way of example, the following reducing agents may be employed: $NaBH(OAc)_3$, sodium borohydride, sodium cyanoborohydride, organic borane complex compounds such as a 4-(dimethylamin)pyridine borane complex, N-ethyl-

diisopropylamine borane complex, N-ethylmorpholine borane complex, N-methylmorpholine borane complex, N-phenylmorpholine borane complex, lutidine borane complex, triethylamine borane complex, or trimethylamine borane complex. $NaBH_4$ and $NaCNBH_3$ are preferred, and $NaCNBH_3$ is particularly preferred.

[0206] In one embodiment, the HAS derivative is added to an aqueous solution containing the biologically active agent. Preferably, subsequently the at least one reducing agent is added, in particular $NaCNBH_3$.

[0207] In an alternative embodiment, the HAS derivative may be optionally brought into an aqueous solution, and then BA is added. Preferably, subsequently the at least one reducing agent is added, in particular $NaCNBH_3$.

[0208] The reaction of the HAS derivative with the amino group of the biologically active compound BA in step (ii) is preferably carried out at a pH value of from 3 to 9, preferably of from 3 to 8, more preferably of from 3 to 7, more preferably from 3 to below 7, such as at a pH of 3 or 4 or 5 or 6. The suitable pH value of the reaction mixture may be adjusted by adding at least one suitable buffer. Among the preferred buffers, sodium acetate buffer, phosphate or borate buffers may be mentioned.

[0209] The reaction of the HAS derivative obtained in step b) with the amino group of the biologically active compound BA in step (ii) is preferably carried out at a temperature of from 0 to 37 °C, more preferably of from 0 to 25 °C, such as 0, 5, 10, 15, 20, or 25 °C.

[0210] The reaction time in step (ii) depends on the temperature, the ratio of HAS, in particular HES, derivative and compound BA and the absolute concentration of the HAS derivative and compound BA. Generally, reaction times from 1 h to 7 d are conceivable.

[0211] The molar ratio of HAS derivative obtained to compound BA in step (ii) is preferably from 0:1 to 200:1 equivalents, even more preferably from 1:1 to 100:1, based on the number average molecular weight (M_n) of the HAS derivative. Preferably, the molar ratio is from 1:1 to 50:1. Low molar ratios such as molar ratios of 50:1 or below, preferably from 1:1 to 20:1, more preferably from 1:1 to 10:1, and even more preferably from 2:1 to 9:1, more preferably from 3:1 to 8:1 and even more preferably from 4:1 to 7:1, are conceivable, for example, if BA is a protein, in particular IFN alpha.

[0212] In a particular preferred embodiment the concentration of the HAS derivative used in step (ii) is higher than about 10 wt.-%, in particular higher than about 15 wt.-%, in each case related to the weight of the reaction solution of (ii).

[0213] Therefore, the present invention also relates to the method as described above, wherein in (ii), the reaction is carried out, preferably in an aqueous system, in the presence of a reducing agent, preferably $NaCNBH_3$, at a temperature in the range of from 0 to 37 °C, preferably 0 to 25 °C and a pH in the range of from 3 to 9, preferably 3 to below, and wherein in (ii), the molar ratio of the HAS derivative to biologically active agent BA is from 0.1:1 to 200:1 equivalents, preferably from 1:1 to 50:1 equivalents, based on the number average molecular weight (M_n) of the HAS derivative.

[0214] Preferred concentrations of BA, such as, e.g., preferred protein concentrations of the solution, preferably the aqueous solution, subjected to (ii) are in the range of from 0.1 to 10 g/l, more preferably from 1 to 9 g/l. The concentration of the HAS derivative in said solution, prior to (ii) and given in (w/v), is preferably in the range of from 0.1 to 50 %, more preferably from 0.5 to 45 %v and more preferably from 1 to 40 %.

[0215] According to a conceivable embodiment, the biologically active agent BA may be dissolved in an aqueous medium, preferably in an aqueous buffer solution, in particular in a sodium acetate buffer solution. The aqueous solution additionally may contain additives, such as detergents and/or dispersants, in particular selected from the group consisting of SDS, Chaps, Tween 20, Tween 80, Nonidet P-40, and Triton X 100. If a detergent and/or dispersant is used, it is preferably present in amount of 0.05 to 3 wt.-%, preferably about 0.5 wt.-%, based on the total weight of the aqueous solution.

[0216] If the at least one reducing agent is employed according to the present invention, and X, present in the HAS derivative employed for the reaction with BA, is, e.g., -CH=N-, -CH=N-O-, or -CH=N-NH-(C=O)- is preferably reduced to result in a functional group X, being $-CH_2-NH-$, $-CH_2-NH-O-$, or $-CH_2-NH-NH-(C=O)-$ under the reductive amination conditions used for the reaction in (ii).

[0217] The present invention thus also relates to the HAS derivative obtained or obtainable by the method as described above, in particular by a method as described above wherein in (ii), the reaction is carried out, preferably in an aqueous system, in the presence of a reducing agent, preferably $NaCNBH_3$, at a temperature in the range of from 0 to 37 °C, preferably 0 to 25 °C and a pH in the range of from 3 to 9, more preferably from 3 to 7, more preferably from 3 to below 7, and wherein in (ii), the molar ratio of the HAS derivative to biologically active agent BA is from 0.1:1 to 200:1 equivalents, preferably from 1:1 to 10:1 equivalents, based on the number average molecular weight (M_n) of the HAS derivative employed in step (ii), in each case with the proviso that X is not an amid group. Particularly preferred molar ratios of the HAS derivative to biologically active agent BA are below 10, such from 1:1 to 9:1, more preferably from 1:1 to 8:1, more preferably from 1:1 to 7:1, more preferably from 1:1 to 6:1 and even more preferably from 1:1 to 5:1, such as about 1:1, about 2:1, about 3:1, about 4:1, or about 5:1. While such low molar ratios like molar ratios of 50:1 or below, more preferably of 10:1 1 or below are conceivable for several biologically active agents, they are preferred for e.g. proteins wherein IFN alpha is especially preferred. As far as, e.g., G-CSF and EPO are concerned, molar ratios of 50:1 or below such as, e.g., from 1:1 to 50:1, are preferred. In general, by way of example, molar ratios in the range of from 1:1 to 50:1 or from 2:1 to 40:1 or from 3:1 to 30:1 or from 4:1 to 20:1 or from 5:1 to 15:1 may be mentioned.

[0218]   In case a protein is employed as biologically active agent BA according to the present invention, and especially at the preferred pH ranges given above, particularly at a pH below 7 and greater or equal 3, to react the HAS derivative predominantly with the amino group located at the N terminus of the protein. The term "predominantly" as used in the context of the present invention relates to an embodiment where at least 80 %, preferably at least 85 % of the N-terminal amino groups available are reacted via reductive amination. It is also possible to react at least 90 % or at least 95 % or at least 96 % or at least 97 % or at least 98 % or at least 99 % of the N-terminal amino groups available. Although coupling to amino groups other than the N-terminal amino group could not be ruled out completely, it is believed that coupling via reductive amination according to the present invention at a pH of below 7, takes place essentially selectively at the N-terminal amino group. In particular, these reaction conditions are preferred for proteins which are stable at these conditions. Should a protein e.g. be acid labile, such as alphal-antitrypsin (AlAT), then it is preferred to chose appropriate reaction conditions, in particular a pH from lower than 7.5 to greater than 5.

[0219]   Therefore, the present invention also relates to a method for the preparation of a HAS derivative comprising BA', a HAS derivative obtainable or obtained by such method, and a HAS derivative according to formula (IV) as such, as described above, wherein the protein comprises the N-terminal amino group and at least one further amino group, said HAS derivative comprising the HAS being predominantly coupled to the N-terminal amino group.

[0220]   Hence, the present invention also relates to a HAS derivative according to formula (IV)

$$(IV)$$
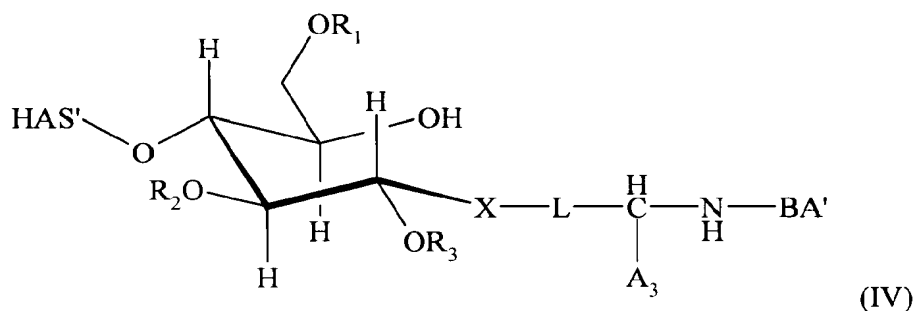
[0221]   Moreover, the present invention also relates to a hydroxyalkyl starch derivative of formula (IV)

$$(IV)$$

wherein X is a functional group resulting from the reaction of an amino group M of a crosslinking compound of formula (II)

M-L-A   (II)

wherein X is not an amide group -C(=O)-NH-,
with hydroxyalkyl starch (HAS) of formula (I)

$$\underset{R_2O}{\overset{H}{\underset{}{\bigvee}}}\quad OR_1$$

via carbon atom C* of the HAS, wherein C* is optionally oxidised prior to the reaction of HAS with M,

wherein HAS' is the remainder of the hydroxyalkyl starch molecule and R_1, R_2 and R_3 are independently hydrogen or a linear or branched hydroxyalkyl group,

wherein A is a residue according to formula (IIa)

$$\begin{array}{c} Z_2A_2 \\ | \\ \text{---}\,C\text{---}Z_1A_1 \\ | \\ A_3 \end{array}$$

wherein

Z_1 and Z_2 are each independently O or S or NR_x, preferably O, wherein R_x is H or lower alkyl such as methyl, ethyl, or propyl such as n-propyl or i-propyl, or C(O)-R_y wherein R_y is preferably selected from the group consisting of C_1-C_6 alkyl and C_6-C_{14} aryl, even more preferably selected from the group consisting of optionally substituted, preferably non-substituted methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, and tert-butyl; R_x preferably being H; A_1 and A_2 are each independently methyl, ethyl, n-propyl, isopropyl, n-butyl, tert-butyl, benzyl, 1,1,1-trichloroethyl, nitrobenzyl, methoxybenzyl, ethoxybenzyl, or are forming a ring according to formula (IIb)

$$\begin{array}{c} Z_2A_2 \\ | \\ \text{---}\,C\text{---}Z_1A_1 \\ | \\ A_3 \end{array}$$

wherein A_1 and A_2, taken together, are -$(CH_2)_2$- or -$(CH_2)_3$- or -$(CH_2CH(CH_3))$-, and wherein A_3 is H or methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, pentyl, benzyl, or is forming a ring with the N atom of the amino group M or with a suitable atom comprised in L, A_3 preferably being H.;

and wherein L is a spacer bridging M and A,

wherein BA' is the remainder of a biologically active agent BA'-NH_2 remaining after the reaction of the amino group of BA via reductive amination with A or with the aldehyde group or keto group corresponding to A.

[0222] As to preferred embodiments with regard to HAS, preferably HES, and the crosslinking compound, reference is made to the respective disclosure above.

[0223] As far as the HAS derivative of formula (IV) is concerned, X is preferably selected from the group consisting of -CH_2-NH-, -CH=N-, -CH_2-NH-O-, and -CH=N-O-, more preferably -CH_2-NH- and -CH_2-NH-O-, most preferably -CH_2-NH-.

[0224] Moreover, as far as the HAS derivative of formula (IV) is concerned, L bridging M and A is preferably a spacer comprising at least one structural unit according to formula (IId), preferably consisting of a structural unit according to formula (IId)

$$\left(\!\!\begin{array}{c} L_1 \\ | \\ C \\ | \\ L_2 \end{array}\!\!\right)_{\!n}$$

wherein L_1 and L_2 are independently from each other H or an organic residue selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, alkylaryl, substituted alkylaryl, and residues -O-R'' wherein R'' is selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, alkylaryl, substituted alkylaryl; preferably H or an organic residue selected from the group consisting of alkyl and substituted alkyl; more preferably H or alkyl; more preferably H,

[0225] wherein n is an integer from 1 to 20, preferably from 1 to 10, more preferably from 1 to 6, more preferably from 1 to 4, more preferably 2.

[0226] The term "biologically active substance" (BA) as used in the context of the present invention relates to a substance which can affect any physical or biochemical property of a biological organism including, but not limited to, viruses, bacteria, fungi, plants, animals, and humans. In particular, the term "biologically active substance" as used in the context of the present invention relates to a substance intended for diagnosis, cure, mitigation, treatment, or prevention of disease in humans or animals, or to otherwise enhance physical or mental well-being of humans or animals. Examples of active substances include, but are not limited to, peptides, polypeptides, proteins, enzymes, small molecule drugs, dyes, lipids, nucleosides, nucleotides, oligonucleotides, polynucleotides, nucleic acids, cells, viruses, liposomes, micro-particles, and micelles. Preferably, a biologically substance according to the present invention contains a native amino group.

[0227] Examples of proteins include, but are not limited to, erythropoietin (EPO), such as recombinant human EPO (rhEPO) or an EPO mimetic, colony-stimulating factors (CSF), such as G-CSF like recombinant human G-CSF (rhG-CSF), alpha-Interferon (IFN alpha), beta-Interferon (IFN beta) or gamma-Interferon (IFN gamma), such as IFN alpha and IFN beta like recombinant human IFN alpha or IFN beta (rhIFN alpha or rhIFN beta), interleukines, e.g. IL-1 to IL-34 such as IL-2 or IL-3 or IL-11 like recombinant human IL-2 or IL-3 (rhIL-2 or rhIL-3), serum proteins such as coagulation factors II-XIII like factor VIII, factor VII, factor IX, serine protease inhibitors such as alphal-antitrypsin (A1AT), activated protein C (APC), plasminogen activators such as tissue-type plasminogen activator (tPA), such as human tissue plasminogen activator (hTPA), AT III such as recombinant human AT III (rhAT III), myoglobin, albumin such as bovine serum albumin (BSA), growth factors, such as epidermal growth factor (EGF), thrombocyte growth factor (PDGF), fibroblast growth factor (FGF), brain-derived growth factor (BDGF), nerve growth factor (NGF), B-cell growth factor (BCGF), brain-derived neurotrophic growth factor (BDNF), ciliary neurotrophic factor (CNTF), transforming growth factors such as TGF alpha or TGF beta, BMP (bone morphogenic proteins), growth hormones such as human growth hormone (hGH), tumor necrosis factors such as TNF alpha or TNF beta, somatostatine, somatotropine, somatomedines, hemoglobin, hormones or prohormones such as insulin, gonadotropin, melanocyte-stimulating hormone (alpha-MSH), triptorelin, hypthalamic hormones such as antidiuretic hormones (ADH and oxytocin as well as releasing hormones and release-inhibiting hormones, parathyroid hormone, thyroid hormones such as thyroxine, thyrotropin, thyroliberin, calcitonin, glucagon, glucagon-like peptides (GLP-1, GLP-2 etc.), exendines such as exendin-4, leptin, vasopressin, gastrin, secretin, integrins, glycoprotein hormones (e.g. LH, FSH etc.), melanoside-stimulating hormones, lipoproteins and apo-lipoproteins such as apo-B, apo-E, apo-L_a, immunoglobulins such as IgG, IgE, IgM, IgA, IgD and fragments thereof, hirudin, tissue-pathway inhibitor, plant proteins such as lectin or ricin, bee-venom, snake-venom, immunotoxins, antigen E, alpha-proteinase inhibitor, ragweed allergen, melanin, oligolysine proteins, RGD proteins or optionally corresponding receptors for one of these proteins; prolactin or a mutant thereof, such as G129R, in which the wild type amino acid at position 129, glycine, is replaced by arginine (a tradename of this mutant is "Lacto Vert") and a functional derivative or fragment of any of these proteins or receptors.

[0228] The polypeptide is preferably selected from the group consisting of erythropoietin (EPO), such as recombinant human EPO (rhEPO), colony-stimulating factors (CSF), such as G-CSF like recombinant human G-CSF (rhG-CSF), alpha-Interferon (IFN alpha), beta-Interferon (IFN beta) gamma-Interferon (IFN gamma), such as IFN alpha and IFN beta like recombinant human IFN alpha or IFN beta (rhIFN alpha or rhIFN beta), factor VII, factor IX, IL-1, IL-11, alphal-antitrypsin (AIAT), and human growth hormone (hGH).

[0229] The active substance is preferably selected from the group composed of antibiotics, antidepressants, antidia-betics, antidiuretics, anticholinergics, antiarrhythmics, antiemetics, antitussives, antiepileptics, antihistamines, antimy-cotics, antisympathotonics, antithrombotics, androgens, antiandrogens, estrogens, antiestrogens, antiosteoporotics, antitumor agents, vasodilators, other antihypertensive agents, antipyretic agents, analgesics, antiinflammatory agents,

beta blockers, immunosuppressants and vitamins.

[0230] Some additional, non-restrictive examples of active substances are alendronate, amikazin, atenolol, azathioprine, cimetidine, clonidine, cosyntropin, cycloserine, desmopressin, dihydroergotamine, dobutamine, dopamine, ε-aminocaproic acid, ergometrine, esmolol, famotidine, flecainide, folic acid, flucytosine, furosemide, ganciclovir, glucagon, hydrazaline, isoproterenol, ketamine, liothyronine, LHRH, merpatricin, methyldopa, metoprolol, neomicin, nimodipine, nystatin, oxytocin, phentolamine, phenylephrine, procainamide, procaine, propranolol, ritodrine, sotalol, terbutaline, thiamine, tiludronate, tolazoline, trimethoprim, tromethamine, vasopressin; amifostine, amiodarone, aminocaproic acid, aminohippurate sodium, aminoglutethimide, aminolevulinic acid, aminosalicylic acid, amsacrine, anagrelide, anastrozole, asparaginase, anthracyclines, bexarotene, bicalutamide, bleomycin, buserelin, busulfan, cabergoline, capecitabine, carboplatin, carmustine, chlorambucin, cilastatin sodium, cisplatin, cladribine, clodronate, cyclophosphamide, cyproterone, cytarabine, camptothecins, 13-cis retinoic acid, all trans retinoic acid; dacarbazine, dactinomycin, daunorubicin, deferoxamine, dexamethasone, diclofenac, diethylstilbestrol, docetaxel, doxorubicin, epirubicin, estramustine, etoposide, exemestane, fexofenadine, fludarabine, fludrocortisone, fluorouracil, fluoxymesterone, flutamide, gemcitabine, epinephrine, L-Dopa, hydroxyurea, idarubicin, ifosfamide, imatinib, irinotecan, itraconazole, goserelin, letrozole, leucovorin, levamisole, lisinopril, lovothyroxine sodium, lomustine, mechlorethamine, medroxyprogesterone, megestrol, melphalan, mercaptopurine, metaraminol bitartrate, methotrexate, metoclopramide, mexiletine, mitomycin, mitotane, mitoxantrone, naloxone, nicotine, nilutamide, octreotide, oxaliplatin, pamidronate, pentostatin, pilcamycin, porfimer, prednisone, procarbazine, prochlorperazine, ondansetron, raltitrexed, sirolimus, streptozocin, tacrolimus, tamoxifen, temozolomide, teniposide, testosterone, tetrahydrocannabinol, thalidomide, thioguanine, thiotepa, topotecan, tretinoin, valrubicin, vinblastine, vincristine, vindesine, vinorelbine, dolasetron, granisetron; formoterol, fluticasone, leuprolide, midazolam, alprazolam, amphotericin B, podophylotoxins, nucleoside antivirals, aroyl hydrazones, sumatriptan; macrolides such as erythromycin, oleandomycin, troleandomycin, roxithromycin, clarithromycin, davercin, azithromycin, flurithromycin, dirithromycin, josamycin, spiromycin, midecamycin, leucomycin, miocamycin, rokitamycin, andazithromycin, and swinolide A; fluoroquinolones such as ciprofloxacin, ofloxacin, levofloxacin, trovafloxacin, alatrofloxacin, moxifloxicin, norfloxacin, enoxacin, grepafloxacin, gatifloxacin, lomefloxacin, sparfloxacin, temafloxacin, pefloxacin, amifloxacin, fleroxacin, tosufloxacin, prulifloxacin, irloxacin, pazufloxacin, clinafloxacin, and sitafloxacin; aminoglycosides such as gentamicin, netilmicin, paramecin, tobramycin, amikacin, kanamycin, neomycin, and streptomycin, vancomycin, teicoplanin, rampolanin, mideplanin, colistin, daptomycin, gramicidin, colistimethate; polymixins such as polymixin B, capreomycin, bacitracin, penems; penicillins including penicillinase-sensitive agents like penicillin G, penicillin V; penicillinase-resistant agents like methicillin, oxacillin, cloxacillin, dicloxacillin, floxacillin, nafcillin; gram negative microorganism active agents like ampicillin, amoxicillin, and hetacillin, cillin, and galampicillin; antipseudomonal penicillins like carbenicillin, ticarcillin, azlocillin, mezlocillin, and piperacillin; cephalosporins like cefpodoxime, cefprozil, ceftbuten, ceftizoxime, ceftriaxone, cephalothin, cephapirin, cephalexin, cephradrine, cefoxitin, cefamandole, cefazolin, cephaloridine, cefaclor, cefadroxil, cephaloglycin, cefuroxime, ceforamide, cefotaxime, cefatrizine, cephacetrile, cefepime, cefixime, cefonicid, cefoperazone, cefotetan, cefinetazole, ceftazidime, loracarbef, and moxalactam, monobactams like aztreonam; and carbapenems such as imipenem, meropenem, pentamidine isethiouate, albuterol sulfate, lidocaine, metaproterenol sulfate, beclomethasone diprepionate, triamcinolone acetamide, budesonide acetonide, fluticasone, ipratropium bromide, flunisolide, cromolyn sodium, and ergotamine tartrate; taxanes such as paclitaxel; SN-38, and tyrphostines.

[0231] Therefore, also chemical compounds known to the skilled person as "small molecules" are conceivable biologically active substances according to the present invention. The term "small molecule" as used in this context of the present invention relates to a biologically active chemical compound other than a protein and an oligonucleotide, including, however, peptides of up to 50 amino acids. Typical examples of such small molecules are listed in the foregoing paragraph.

[0232] Examples for an oligonucleotide are aptamers. Also to be mentioned are peptide nucleic acids (PNA) as conceivable biologically active substances.

[0233] Therefore, the present invention also relates to a method as described above and a HAS derivative as described above, wherein the protein is erythropoietin (EPO) such as recombinant human EPO (rhEPO), a colony-stimulating factor (CSF) such as G-CSF like recombinant human G-CSF (rhG-CSF), interferon (IFN), preferably IFN alpha, IFN beta, IFN gamma, like recombinant human IFN alpha (rhIFN alpha) or recombinant human IFN beta (rhIFN beta), factor VII, factor IX, interleukine-2, interleukine-11, alpha-1-antitrypsin, an antibody, or an antibody fragment, or an alternative protein scaffold.

[0234] The term "alternative protein scaffold" as used in the context of the present invention relates to a molecule having binding abilities similar to a given antibody wherein the molecule is based on an alternative non-antibody protein framework. In this context, the articles by A. Skerra, T. Hey et al., and H.K. Binz (see list of references below) may be mentioned.

[0235] As far as the biologically active substances (BA) of the present invention are concerned, these compounds may comprise one or more amino groups for coupling according to stage (ii) of the present invention. For cases where BA as such does not comprise an amino group suitable for this coupling, it is conceivable that at least one such amino group is introduced into BA by suitable functionalisation via methods known to the skilled person, prior to subjecting BA

to (ii).

[0236] In accordance with above-described preferred crosslinking compounds and the HAS derivatives obtained there-from, the following HAS derivatives may be mentioned as preferred embodiments by way of example, wherein in each case, HAS is - according to preferred embodiments of the present invention - HES:

or

or

or
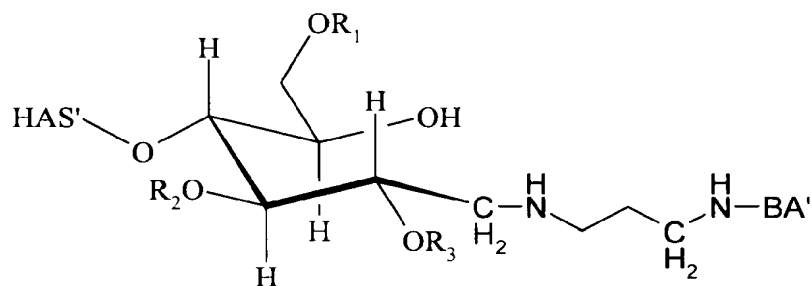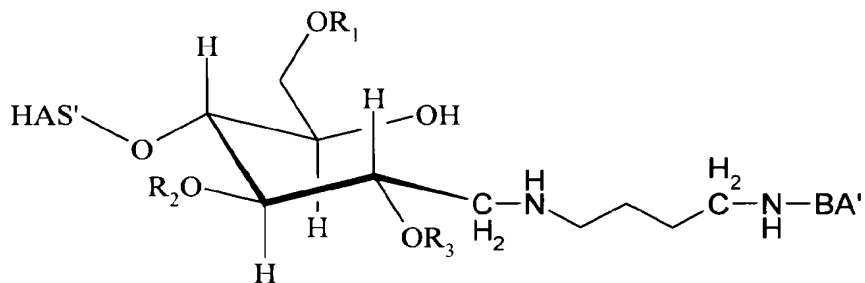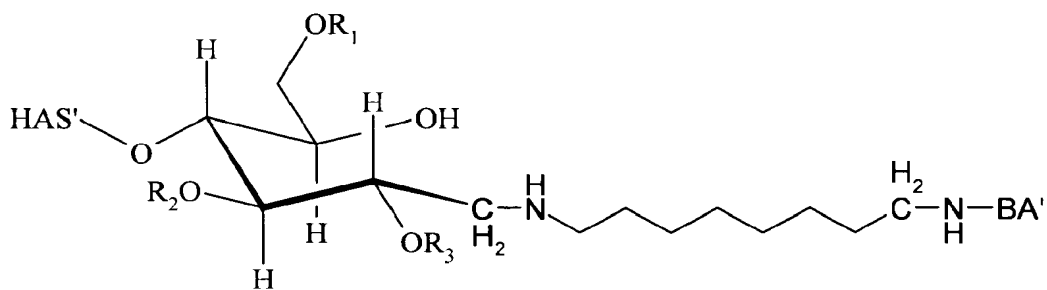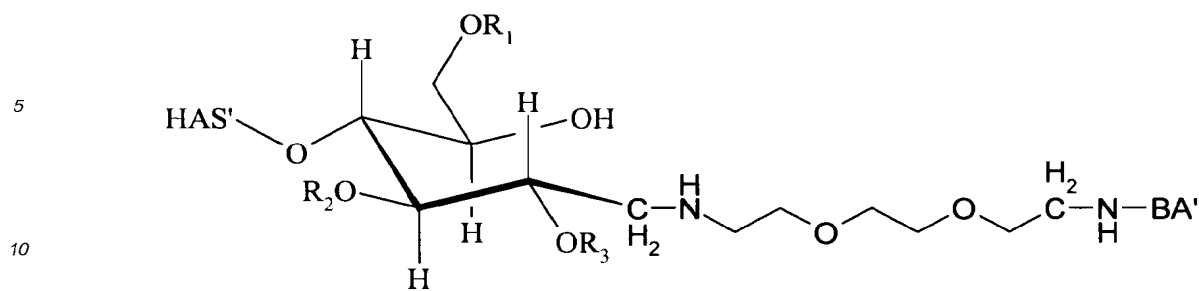
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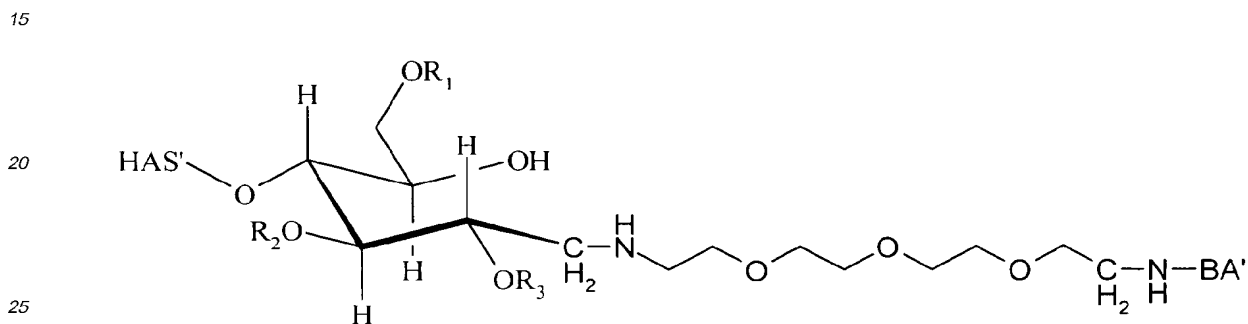
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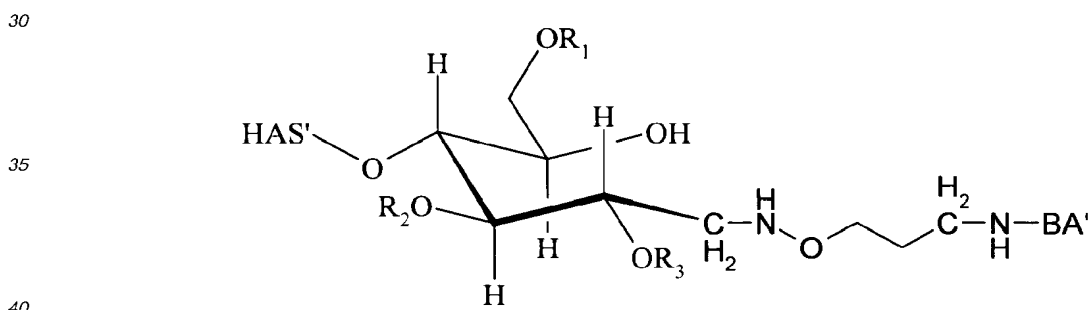
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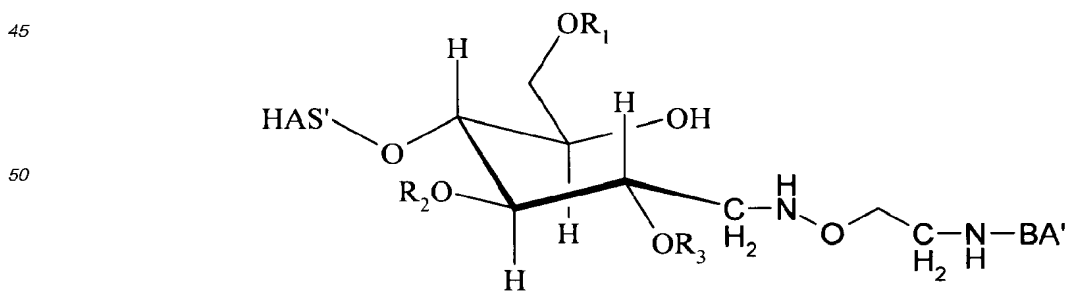
wherein BA' is a protein, more preferably, wherein the protein is erythropoietin (EPO) such as recombinant human EPO (rhEPO), a colony-stimulating factor (CSF) such as G-CSF like recombinant human G-CSF (rhG-CSF), interferon (IFN), preferably IFN alpha, IFN beta, IFN gamma, like recombinant human IFN alpha (rhIFN alpha) or recombinant human

IFN beta (rhIFN beta), factor VII, factor IX, interleukine-2, interleukine-11, alpha-1-antitrypsin, an antibody, or an antibody fragment, or an alternative protein scaffold, in particular, wherein the protein is EPO, IFN alpha or GCSF. Even more preferably, HAS' is HES', wherein, even more preferably, HES has a mean molecular weight of about 1 to 500 kDa, preferably 10 to 150 kDa, a molar substitution of 0.1 to 3, preferably 0.4 to 1.3, such as 0.4, 0.5, 0.6, 0.7 0.8, 0.9, 1.0, 1.1, 1.2, or 1.3, preferably of 0.7 to 1.3, such as 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, or 1.3, most preferably about 1.0, 1.1, 1.2 or 1.3, and a ratio of $C_2 : C_6$ substitution of preferably in the range of from 2 to 20, more preferably in the range of from 2 to 15 and even more preferably in the range of from 3 to 12.

[0237] According to especially preferred embodiments, the present invention relates to a HAS derivative according to formula

wherein HAS is preferably HES and wherein, even more preferably, HES has a mean molecular weight from about 1 to about 500 kDa, more preferably from about 2 to 400 kDa, more preferably from about 5 to about 300 kDa, more preferably from about 10 to about 200 kDa, in particular from about 50 to about 150 kDa, a molar substitution of 0.1 to 3, preferably 0.4 to 1.3, such as 0.4, 0.5, 0.6, 0.7 0.8, 0.9, 1.0, 1.1, 1.2, or 1.3, and a ratio of $C_2 : C_6$ substitution of preferably in the range of from 2 to 20, more preferably in the range of from 2 to 15 and even more preferably in the range of from 3 to 12.

[0238] According to a further especially preferred embodiments, the present invention relates to a HAS derivative according to formula

wherein HAS is preferably HES and wherein, even more preferably, HES has a mean molecular weight from about 1 to about 500 kDa, more preferably from about 2 to 400 kDa, more preferably from about 5 to about 300 kDa, more preferably from about 10 to about 200 kDa, in particular from about 50 to about 150 kDa, a molar substitution of 0.1 to 3, preferably 0.4 to 1.3, such as 0.4, 0.5, 0.6, 0.7 0.8, 0.9, 1.0, 1.1, 1.2, or 1.3, and a ratio of $C_2 : C_6$ substitution of preferably in the range of from 2 to 20, more preferably in the range of from 2 to 15 and even more preferably in the range of from 3 to 12.

[0239] According to a further especially preferred embodiments, the present invention relates to a HAS derivative according to formula

wherein HAS is preferably HES and wherein, even more preferably, HES has a mean molecular weight from about 1 to about 500 kDa, more preferably from about 2 to 400 kDa, more preferably from about 5 to about 300 kDa, more preferably from about 10 to about 200 kDa, in particular from about 50 to about 150 kDa, a molar substitution of 0.1 to 3, preferably 0.4 to 1.3, such as 0.4, 0.5, 0.6, 0.7 0.8, 0.9, 1.0, 1.1, 1.2, or 1.3, and a ratio of $C_2 : C_6$ substitution of preferably in the range of from 2 to 20, more preferably in the range of from 2 to 15 and even more preferably in the range of from 3 to 12.

[0240] According to a further aspect, the present invention relates to a HAS derivative comprising BA' as described above, or a HAS derivative comprising BA' as described above, obtained or obtainable by a method as described above, for use in a method for the treatment of the human or animal body.

[0241] Moreover, the present invention relates to a HAS derivative comprising BA' as described above, or a HAS derivative comprising BA' as described above, obtained or obtainable by a method as described above, as a therapeutic or prophylactic agent.

[0242] Furthermore, the present invention relates to a pharmaceutical composition comprising in a therapeutically effective amount a HAS derivative comprising BA' as described above, or a HAS derivative comprising BA' as described above, obtained or obtainable by a method as described above.

[0243] The HAS derivatives of the present invention, comprising BA', may be administered by suitable methods such as e.g. enteral, parenteral or pulmonary methods preferably administered by i.v., s.c. or i.m. routes. The specific route chosen will depend upon the condition being treated. Preferably, the derivatives may be administered together with a suitable carrier, such as known in the art (e.g. as used in the first generation/unmodified biopharmaceutical, albumin-free or with albumin as an excipient), a suitable diluent, such as sterile solutions for i.v., i.m., or s.c. application. The required dosage will depend on the severity of the condition being treated, the patient's individual response, the method of administration used, and the like. The skilled person is able to establish a correct dosage based on his general knowledge.

[0244] As far as the pharmaceutical compositions according to the present invention comprising the HAS derivative comprising BA', as described above, are concerned, the HAS derivatives may be used in combination with a pharmaceutical excipient. Generally, the HAS derivative will be in a solid form which can be combined with a suitable pharmaceutical excipient that can be in either solid or liquid form. As excipients, carbohydrates, inorganic salts, antimicrobial agents, antioxidants, surfactants, buffers, acids, bases, and combinations thereof may be mentioned. A carbohydrate such as a sugar, a derivatized sugar such as an alditol, aldonic acid, an esterified sugar, and/or a sugar polymer may be present as an excipient. Specific carbohydrate excipients include, for example: monosaccharides, such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol, sorbitol (glucitol), pyranosyl sorbitol, myoinositol, and the like. The excipient may also include an inorganic salt or buffer such as citric acid, sodium chloride, potassium chloride, sodium sulfate, potassium nitrate, sodium phosp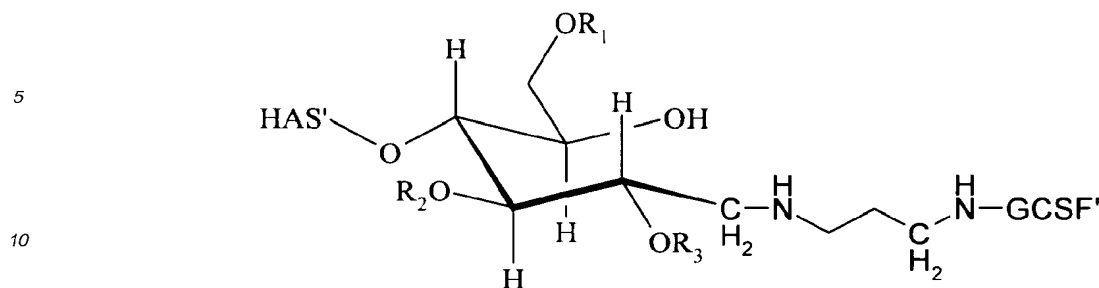hate monobasic, sodium phosphate dibasic, and combinations thereof. The pharmaceutical composition according to the present invention may also comprise an antimicrobial agent for preventing or deterring microbial growth, such as, e.g., benzalkonium chloride, benzethonium chloride, benzyl alcohol, cetylpyridinium chloride, chlorobutanol, phenol, phenylethyl alcohol, phenylmercuric nitrate, thimersol, and combinations thereof. The pharmaceutical composition according to the present invention may also comprise an antioxidant. such as, e.g., ascorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, hypophosphorous acid, monothioglycerol, propyl gallate, sodium bisulfite, sodium formaldehyde sulfoxylate, sodium metabisulfite, and combinations thereof. The pharmaceutical composition according to the present invention may also comprise a surfactant may such as, e.g., polysorbates, or pluronics sorbitan esters; lipids, such as phospholipids such as lecithin and other phosphatidylcholines, phosphatidylethanolamines acids and fatty esters; steroids, such as cholesterol; and chelating agents, such as EDTA or zinc. The pharmaceutical composition according to the present invention may also comprise acids or bases such as, e.g., hydrochloric acid, acetic acid, phosphoric acid, citric acid, malic acid, lactic acid, formic acid, trichloroacetic acid,

nitric acid, perchloric acid, phosphoric acid, sulfuric acid, fumaric acid, and combinations thereof, and/or sodium hydroxide, sodium acetate, ammonium hydroxide, potassium hydroxide, ammonium acetate, potassium acetate, sodium phosphate, potassium phosphate, sodium citrate, sodium formate, sodium sulfate, potassium sulfate, potassium fumerate, and combinations thereof. Generally, the excipient will be present in pharmaceutical composition according to the present invention in an amount of 0.001 to 99.999 wt.-%, preferably from 0.01 to 99.99 wt.-%, more preferably from 0.1 to 99.9 wt.-%, in each case based on the total weight of the pharmaceutical composition.

[0245] According to another aspect, the present invention also relates to the use of a HAS derivative, preferably a HES derivative as described above, wherein BA is EPO, for the preparation of a medicament for the treatment of anemic disorders or hematopoietic dysfunction disorders or diseases related hereto.

[0246] According to another aspect, the present invention also relates to the use of a HAS derivative, preferably a HES derivative as described above, wherein BA is G-CSF, for the preparation of a medicament for the treatment of haemophilia A for the treatment of a disorder characterized by a reduced hematopoietic or immune function. According to a preferred embodiment, the disorder characterized by a reduced hematopoietic or immune function, is a result of chemotherapy, radiation therapy, infectious disease, severe chronic neutropenia, or leukemia.

[0247] According to another aspect, the present invention also relates to the use of a HAS derivative, preferably a HES derivative as described above, wherein BA is IFN alpha, for the preparation of a medicament for the treatment of leukaemia e.g. hairy cell leukaemia, chronic myelogeneous leukaemia, multiple myeloma, follicular lymphoma, cancer, e.g. carcinoid tumour, malignant melanoma and hepatitis, e.g. chronic hepatitis B and chronic hepatitis C.

[0248] According to another aspect, the present invention also relates to the use of a HAS derivative, preferably a HES derivative as described above, wherein BA is IFN gamma, for the preparation of a medicament for the treatment of osteoporosis and/or chronic malignant disease.

[0249] According to another aspect, the present invention also relates to the use of a HAS derivative, preferably a HES derivative as described above, wherein BA is IL-2, for the preparation of a medicament for the treatment of osteoporosis and/or chronic malignant disease.

[0250] According to another aspect, the present invention also relates to the use of a HAS derivative, preferably a HES derivative as described above, wherein BA is IL-11, for the preparation of a medicament for the treatment of platelet transfusions following myelosuppressive chemotherapy.

[0251] According to another aspect, the present invention also relates to the use of a HAS derivative, preferably a HES derivative as described above, wherein BA is A1AT, for the preparation of a medicament for the treatment of emphysema, cystic fibrosis, atopic dermatitis, and/or bronchitis.

[0252] According to another aspect, the present invention also relates to the use of a HAS derivative, preferably a HES derivative as described above, wherein BA is IFN beta, for the preparation of a medicament for the treatment of multiple sclerosis, preferably relapsing forms of multiple sclerosis.

[0253] According to another aspect, the present invention also relates to the use of a HAS derivative, preferably a HES derivative as described above, wherein BA is factor VII for the preparation of a medicament for the treatment of episodes in hemophilia A or B patients with inhibitors to Factor VIII or Factor IX.

[0254] According to another aspect, the present invention also relates to the use of a HAS derivative, preferably a HES derivative as described above, wherein BA is factor IX for the preparation of a medicament for the control and prevention of hemorrhagic episodes in patients with hemophilia B, e.g. congenital factor IX deficiency or Christmas disease, including control and prevention of bleeding in surgical settings.

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Description of the Figures

[0256]   Figure 1: SDS-PAGE analysis of an oxHES55/0.7-IFNa coupling reaction
Figure 1 shows the SDS-PAGE analysis of an oxHES-IFNa coupling reactions according to example 2. The separation was performed under reducing conditions using the NuPAGE system (Invitrogen) with 4-12% Bis-Tris gels (1.0 mm) and a MOPS running buffer according to the manufacturers instructions.

Load: 10 µg protein as reaction mix.
M: Marker, Mark12 (Invitrogen).
Lanes 1-4: reaction mixtures according to example 2.
Successful HESylation of the target protein (19 kDa) becomes visible as a smeary band with a broad mass distribution ranging from 30 to >200 kDa.

Figure 2: Anion exchange chromatography of an oxHES55/0.7-IFNa coupling reaction
Figure 2 shows the chromatographic separation monitored by UV-Vis spectroscopy at 221 nm on an ion exchange column of an oxHES55/0.7-IFNa coupling reaction according to example 2. Chromatography conditions were as follows:
Chromatography system: Äkta Explorer 100 (GE Healthcare).
Column: Hi Trap Q HP 1 ml (GE Healthcare)
Eluent A: 10 mM Tris·Cl, pH 8.0.
Eluent B: 10 mM Tris·Cl, 0.5 M NaCl, pH 8.0.
Operating conditions: flow rate 1 ml/min, 21 °C.
Run parameters:

equilibration	10 CV	0%B
sample load		
wash	2 CV	0%B
elution	16 CV	0-50%B
regeneration	10 CV	100%B
reequilibration	8 CV	0%B

Load: 2 mg protein/ml resin as reaction mix according to example 2, 20fold diluted in Eluent A and adjusted to pH 8.0. Non-reacted, excessive HES is found in the flowthrough. The HESylation weakens the interaction of the protein with the column resulting in decreased elution times for the conjugate as compared to the unmodified protein.

Figure 3: SDS-PAGE analysis of an oxHES55/0.7-EPO coupling reaction
Figure 3 shows the SDS-PAGE analysis of an oxHES55/0.7-EPO coupling reaction according to example 3. The separation was performed under reducing conditions using the NuPAGE system (Invitrogen) with 4-12% Bis-Tris gels (1.0 mm) and a MOPS running buffer according to the manufacturers instructions. Load: 10 μg protein as reaction mixture.

M: Marker, Mark 12 (Invitrogen).
Lane 1: reaction mixture according to example 3.
Lane 2: EPO starting material prior to conjugation.
Successful HESylation of the target protein (□35-40 kDa) becomes visible as a smeary band with a broad mass distribution ranging from 55 to >200 kDa.

Figure 4: Cation exchange chromatography of an oxHES55/0.7-EPO coupling reaction
Figure 4 shows the chromatographic separation monitored by UV-Vis spectroscopy at 221 nm on an ion exchange column of an oxHES55/0.7-EPO coupling reaction according to example 3. Chromatography conditions were as follows:
Chromatography system: Äkta Explorer 100 (GE Healthcare).
Column: HiTrap SP HP (GE Healthcare).
Eluent A: 20 mM sodium acetate, pH 4.0.
Eluent B: 20 mM sodium acetate, 1M NaCl, pH 4.0.
Operating conditions: flow rate 5 ml/min, 21 °C.
Run parameters:

equilibration sample load	10 CV	0%B
wash1	2 CV	0%B
wash2	2 CV	10%B
elution	21 CV	10-52%B
regeneration	8 CV	100%B
reequilibration	5 CV	0%B

Load: 2 mg protein/ml resin as reaction mix according to example 3, 10fold diluted in Eluent A
Non-reacted, excessive HES is found in the flowthrough. The HESylation weakens the interaction of the protein with the column resulting in decreased elution times for the conjugate as compared to the unmodified protein.

Figure 5: SDS-PAGE analysis of an oxHES100/1.0-IFNa coupling reaction
Figure 5 shows the SDS-PAGE analysis of a oxHES-IFNa coupling reactions according to example 5. The separation was performed under reducing conditions using the NuPAGE system (Invitrogen) with 4-12% Bis-Tris gels (1.0 mm) and a MOPS running buffer according to the manufacturers instructions.

Load: 10 μg protein as reaction mix.
M: Marker, Mark12 (Invitrogen).
lane 1: reaction mixture according to example 5.
Successful HESylation of the target protein (19 kDa) becomes visible as a smeary band with a broad mass distribution ranging from 50 to >200 kDa.

Figure 6: Anion exchange chromatography of an oxHES100/1.0-IFNa coupling reaction
Figure 6 shows the chromatographic separation monitored by UV-Vis spectroscopy at 221 nm on an ion exchange column of an oxHES100/1.0-IFNa coupling reaction according to example 5. Chromatography conditions were as follows:
Chromatography system: Äkta Explorer 100 (GE Healthcare).
Column: Hi Trap Q HP 5 ml (GE Healthcare).
Eluent A: 10 mM Tris·Cl, pH 8.0.
Eluent B: 10 mM Tris·Cl, 0.5 M NaCl, pH 8.0.
Operating conditions: flow rate 1 ml/min, 21 °C.
Run parameters:

equilibration sample load	10 CV	0%B
wash	2 CV	0%B
elution	12.5 CV	0-50%B

(continued)

regeneration	5 CV	100%B
reequilibration	5 CV	0%B

Load: 2 mg protein/ml resin as reaction mix according to example 5, 20fold diluted in Eluent A and adjusted to pH 8.0
Non-reacted, excessive HES is found in the flowthrough. The HESylation weakens the interaction of the protein with the column resulting in decreased elution times for the conjugate as compared to the unmodified protein.

Figure 7: Peptide Mapping of an oxHES100/1.0-IFNa conjugate

Figure 7 shows the chromatographic separation of an IEX-purified oxHES-IFNa conjugate according to example 5 treated with Endo-LysC.

The proteolysis was performed using 7.5% Endo-LysC in 50 mM Tris-Cl, pH 8.6, 0.01 % SDS at 37 °C o/n. Samples were denatured with DTT and guanidinium chloride and analyzed by RP-HPLC on a 4.6 x 250 mm Jupiter C4 column (Phenomenex) run with a water/acetonitrile gradient with TFA. The chromatograms shown were monitored at 214 nm.

The arrow indicates the region of the chromatogram where strong differences between the chromatograms for the protein (A) and the conjugate (B) are visible. The peaks for L1 and L1/L2 (the N-terminal peptide resulting from the Endo-Lys C treatment) is strongly reduced for the conjugate sample while the other fragments remain virtually unaffected. These data suggest a preferential coupling of the HES to the N-terminus of IFNa.

Figure 8: SDS-PAGE analysis of an oxHES100/1.0-EPO coupling reaction

Figure 8 shows the SDS-PAGE analysis of an oxHES100/1.0-EPO coupling reaction according to example 6. The separation was performed under reducing conditions using the NuPAGE system (Invitrogen) with 4-12% Bis-Tris gels (1.0 mm) and a MOPS running buffer according to the manufacturers instructions.

Load: 10 μg protein as reaction mixture.
M: Marker, Mark12 (Invitrogen).
Lane 1: 10 μg EPO starting material prior to conjugation.
Lane 2: 5 μg EPO starting material prior to conjugation.
Lane 3: reaction mixture according to example 6.
Successful HESylation of the target protein (□35-40 kDa) becomes visible as a smeary band with a broad mass distribution ranging from 70 to >200 kDa.

Figure 9: Cation exchange chromatography of an oxHES100/1.0-EPO coupling reaction

Figure 14 shows the chromatographic separation monitored by UV-Vis spectroscopy at 221 nm on an ion exchange column of an oxHES100/1.0-EPO coupling reaction according to example 6. Chromatography conditions were as follows:
Chromatography system: Äkta Explorer 100 (GE Healthcare).
Column: 2x 5 ml HiTrap SP HP (GE Healthcare).
Eluent A: 20 mM sodium acetate, pH 4.0.
Eluent B: 20 mM sodium acetate, 1 M NaCl, pH 4.0.
Operating conditions: flow rate 5 ml/min, 21 °C.
Run parameters:

equilibration	10 CV	0%B
sample load		
wash1	2 CV	0%B
wash2	2 CV	10%B
elution	21 CV	10-52%B
regeneration	2.5 CV	100%B
reequilibration	5 CV	0%B

Load: 2 mg protein/ml resin as reaction mix according to example 6, 10fold diluted in Eluent A
Non-reacted, excessive HES is found in the flowthrough. The HESylation weakens the interaction of the protein with the column resulting in decreased elution times for the conjugate as compared to the unmodified protein.

Figure 10: SDS-PAGE analysis of an oxHES100/1.0-G-CSF coupling reaction

Figure 10 shows the SDS-PAGE analysis of an oxHES100/1.0- G-CSF coupling reaction according to example 7. The separation was performed under reducing conditions using the NuPAGE system (Invitrogen) with 4-12% Bis-Tris gels (1.0 mm) and a MOPS running buffer according to the manufacturers instructions.

Load: 10 μg protein as reaction mixture.
M: Marker, Mark12 (Invitrogen).
Lane 1: reaction mixture according to example 7.
Lane 2: G-CSF starting material prior to conjugation.
Successful HESylation of the target protein (□18 kDa) becomes visible as a smeary band with a broad mass distribution ranging from 50 to >200 kDa.

Figure 11: RP-HPLC analysis of an oxHES100/1.0-G-CSF coupling reaction

Figure 11 shows a section of the RP-HPLC analysis of an oxHES100/1.0-G-CSF coupling reaction according to example 7 monitored by UV-Vis spectroscopy at 221 nm.

Chromatography conditions were as follows:
Chromatography system: Summit, P580 (HPG) (Dionex).
Column: Jupiter C 18, 300A, 5 μm, 4.6 x 150 mm (Phenomenex).
Eluent A: 0.1 % trifluoroacetic acid in water.
Eluent B: 0.1 % trifluoroacetic acid in acetonitrile.
Operating conditions: flow rate 1 ml/min, 20°C.
Gradient: 0-5 min, 5-55%B; 5-12 min, 55-68%B; 12-17 min, 100%B; 17-22 min, 5%B; gradient delay 2.5 min.
Load: 10 μg protein as reaction mix, diluted in water to a protein concentration of 0.1 mg/ml.
The main peak at 11.5 min is the HES protein conjugate separated from free G-CSF eluting at □13 min.

Figure 12: SDS-PAGE analysis of an HES100/1.0-IFNa coupling reaction

Figure 12 shows the SDS-PAGE analysis of a HES-IFNa coupling reactions according to example 10. The separation was performed under reducing conditions using the NuPAGE system (Invitrogen) with 4-12% Bis-Tris gels (1.0 mm) and a MOPS running buffer according to the manufacturers instructions.

Load: 10 μg protein as reaction mixture.
M: Marker, unstained protein marker 5-200 kDa (Serva).
Lane 1: reaction mixture according to example 9.
Successful HESylation of the target protein (19 kDa) becomes visible as a smeary band with a broad mass distribution ranging from 50 to >200 kDa.

Figure 13: Anion exchange chromatography of an HES100/1.0-IFNa coupling reaction

Figure 13 shows the chromatographic separation monitored by UV-Vis spectroscopy at 221 nm on an ion exchange column of an HES100/1.0-IFNa coupling reaction according to example 10. Chromatography conditions were as follows:
Chromatography system: Äkta Explorer 100 (GE Healthcare).
Column: 5 ml HiTrap SP HP (GE Healthcare).
Eluent A: 20 mM sodium acetate, pH 4.0.
Eluent B: 20 mM sodium acetate, 1M NaCl, pH 4.0.
Operating conditions: flow rate 5 ml/min, 21°C.
Run parameters:

equilibration sample load	10 CV	0%B
wash 1	2 CV	0%B
elution	20 CV	0-50%B
regeneration	10 CV	100%B
reequilibration	5 CV	0%B

Load: 3 mg protein/ml resin as reaction mix according to example 9, 10fold diluted in Eluent A
Non-reacted, excessive HES is found in the flowthrough. The HESylation weakens the interaction of the protein with the column resulting in decreased elution times for the conjugate as compared to the unmodified protein.

Figure 14: SDS-PAGE analysis of an HES100/1.0-EPO coupling reaction

Figure 14 shows the SDS-PAGE analysis of an HES100/1.0-EPO coupling reaction according to example 11. The separation was performed under reducing conditions using the NuPAGE system (Invitrogen) with 4-12% Bis-Tris gels (1.0 mm) and a MOPS running buffer according to the manufacturers instructions.

Load: 10 μg protein as reaction mixture.

M: Marker, Mark12 (Invitrogen).
Lane 1: IEX purified HES EPO conjugate.
Lane 2: reaction mixture according to example 11.
Lane 3: 5 μg EPO starting material prior to conjugation.
Successful HESylation of the target protein (□35-40 kDa) becomes visible as a smeary band with a broad mass distribution ranging from 70 to >200 kDa.

Figure 15: Cation exchange chromatography of an HES100/1.0-EPO coupling reaction
Figure 15 shows the chromatographic separation monitored by UV-Vis spectroscopy at 221 nm on an ion exchange column of an HES100/1.0-EPO coupling reaction according to example 11. Chromatography conditions were as follows:
Chromatography system: Äkta Explorer 100 (GE Healthcare).
Column: 4 x 5 ml HiTrap SP HP (GE Healthcare).
Eluent A: 20 mM sodium acetate, pH 4.0.
Eluent B: 20 mM sodium acetate, 1M NaCl, pH 4.0.
Operating conditions: flow rate 5 ml/min, 21 °C.
Run parameters:

equilibration	5 CV	0%B
sample load wash1	2 CV	0%B
elution	13 CV	0-52%B
regeneration	2.5 CV	100%B
reequilibration	2.5 CV	0%B

Load: 3 mg protein/ml resin as reaction mix according to example 11, 2fold diluted in Eluent A.
Non-reacted, excessive HES is found in the flowthrough. The HESylation weakens the interaction of the protein with the column resulting in decreased elution times for the conjugate as compared to the unmodified protein.
Figure 16: SDS-PAGE analysis of an HES100/1.0-G-CSF coupling reaction
Figure 16 shows the SDS-PAGE analysis of an HES100/1.0-G-CSF coupling reaction according to example 12. The separation was performed under reducing conditions using the NuPAGE system (Invitrogen) with 4-12% Bis-Tris gels (1.0 mm) and a MOPS running buffer according to the manufacturers instructions.

Load: 10 μg protein as reaction mixture.
M: Marker, Mark12 (Invitrogen).
Lane 1: IEX purified HES G-CSF conjugate.
Lane 2: reaction mixture according to example 12.
Lane 3: 5 μg G-CSF starting material prior to conjugation.
Successful HESylation of the target protein (□18 kDa) becomes visible as a smeary band with a broad mass distribution ranging from 50 to >200 kDa.

Figure 17: RP-HPLC analysis of an HES100/1.0-G-CSF coupling reaction
Figure 17 shows a section of the RP-HPLC analysis of an HES100/1.0-G-CSF coupling reaction according to example 12 monitored by UV-Vis spectroscopy at 221 nm. Chromatography conditions were as follows:

Chromatography system: Summit, P580 (LPG) (Dionex).
Column: Jupiter C18, 300A, 5 μm, 4.6 x 150 mm (Phenomenex).
Eluent A: 0.1 % trifluoroacetic acid in water.
Eluent B: 0.1 % trifluoroacetic acid in acetonitrile.
Operating conditions: flow rate 1 ml/min, 20°C.
Gradient: 0-5 min, 5-55%B; 5-12 min, 55-68%B; 12-17 min, 100%B; 17-22 min, 5%B; gradient delay 2.5 min.
Load: 10 μg protein as reaction mixture, diluted in water to a protein concentration of 0.1 mg/ml.
The main peak at 10-10.5 min is the HES protein conjugate separated from free G-CSF eluting at □12 min.

Figure 18: Cation exchange chromatography of an HES100/1.0-G-CSF coupling reaction
Figure 18 shows the chromatographic separation monitored by UV-Vis spectroscopy at 221 nm on an ion exchange column of an HES 100/1.0-G-CSF coupling reaction according to example 12. Chromatography conditions were as follows:
Chromatography system: Äkta Explorer 100 (GE Healthcare).

Column: 2 x 5 ml HiTrap SP HP (GE Healthcare).
Eluent A: 20 mM sodium acetate, pH 4.0.
Eluent B: 20 mM sodium acetate, 1M NaCl, pH 4.0.
Operating conditions: flow rate 5 ml/min, 21 °C.
Run parameters:

equilibration	5 CV	0%B
sample load wash	2 CV	0%B
elution	15 CV	0-40%B
regeneration	5 CV	100%B
reequilibration	5 CV	0%B

Load: 3 mg protein/ml resin as reaction mix according to example 12, 2fold diluted in Eluent A.
Non-reacted, excessive HES is found in the flowthrough. The HESylation weakens the interaction of the protein with the column resulting in decreased elution times for the conjugate as compared to the unmodified protein.
Figures 19-23 are referred to in the context of the respective examples.
Figure 24
Figure 24 shows a section of the HPGPC analysis of an oxHBS-BSA coupling reaction according to "additional data (A. 2)" monitored by UV-Vis spectroscopy at 280 nm. Chromatography conditions were as follows:

Chromatography system: Shimadsu LC10 AD/ UV-Detektor: TSP UV 2000
Column: Superose 6 10/300 GL (Pharmacia).
Eluent: Phosphate buffer: (3.887g Na_2HPO_4 x 2 H_2O, 1.967g NaH_2PO_4 x 2 H2O, 11.688g NaCl, 0.05g NaN_3 were dissolved in water for chromatography (Reagent Pharmakopoea Europaea) up to a total volume of 1.0 1. The solution was filtered utilizing a 0.45 μm filter)
Operating conditions: flow rate 0.4 ml/min, 20°C.
Load: 0.9 mg protein as reaction mixture, dissolved in 100 μl to a protein concentration of 9 mg/ml.
The upper part shows the BSA starting material prior to the coupling reaction. From left to right, the peaks are at 38.038, 39.277, and 42.272.
The lower part shows the HBS-BSA conjugate. From left to right, the peaks are at 36.795, 39.345, and 41.521.

Figure 25: SDS-PAGE analysis of an oxHBS-IFNa coupling reaction
Figure 25 shows the SDS-PAGE analysis of an oxHBS-IFNa coupling reaction according to "additional data (A.3)". The separation was performed under reducing conditions using the NuPAGE system (Invitrogen) with 4-12% Bis-Tris gels (1.0 mm) and a MOPS running buffer according to the manufacturers instructions.

Load: 10 μg protein as reaction mixture.
M: Marker, Mark12 (Invitrogen).
Lanes 1-4: reaction mixtures according to "additional data (A.3)".
Successful HBSylation of the target protein (19 kDa) becomes visible as a smeary band with a broad mass distribution ranging from 30 to >200 kDa.

Figure 26: Anion exchange chromatography of an oxHBS-IFNa coupling reaction
Figure 26 shows the chromatographic separation monitored by UV-Vis spectroscopy at 221 nm of an oxHBS-IFNa coupling reaction according to additional data (A.3) using an ion exchange column. Chromatography conditions were as follows:
Chromatography system: Äkta Explorer 100 (GE Healthcare).
Column: Hi Trap Q HP 1 ml (GE Healthcare).
Eluent A: 10 mM Tris·Cl, pH 8.0.
Eluent B: 10 mM Tris·Cl, 0.5 M NaCl, pH 8.0.
Operating conditions: flow rate 1 ml/min, 20°C.
Run parameters:

equilibration	10 CV	0%B
sample load wash	2 CV	0%B
elution	16 CV	0-50%B

(continued)

regeneration	10 CV	100%B
reequilibration	8 CV	0%B

Load: reaction mixture according to example 3, 20fold diluted in Eluent A and adjusted to pH 8.0.

Non-reacted, excessive HBS is found in the flowthrough. The HBSylation weakens the interaction of the protein with the column resulting in decreased elution times for the conjugate as compared to the unmodified protein.

Figure 27: SDS-PAGE analysis of an oxHBS-EPO coupling reaction

Figure 27 shows the SDS-PAGE analysis of an oxHBS-EPO coupling reaction according to "additional data (A.4)". The separation was performed under reducing conditions using the NuPAGE system (Invitrogen) with 4-12% Bis-Tris gels (1.0 mm) and a MOPS running buffer according to the manufacturers instructions.

Load: 10 μg protein as reaction mixture.

M: Marker, Mark12 (Invitrogen).

Lane 1: reaction mixture according to "additional data (A.4)".

Lane 2: EPO starting material prior to conjugation.

Successfull HBSylation of the target protein (□35-40 kDa) becomes visible as a smeary band with a broad mass distribution ranging from 45 to >200 kDa.

Examples

Example 1: Preparation of oxHES55/0.7-N-(3-Propioaldehydediethylacetal)

[0257] HES aldonic acid (oxHES) was synthesized as described in example 9 of WO 2005/083103 A (in said document, the preparation is described for a hyperbranched starch, HBS) starting from HES with a molecular weight of 55 kDa and a molar substitution of 0.7 (HES55/0.7).

30g oxHES 55/0.7, dried for 2d at 80°C, were dissolved in 60 ml dry dimethylformamide (DMF) and the solution was heated to 70°C. 25g 1-amino-3,3-diethoxypropane in 50 ml dry DMF were added and the reaction mixture was heated at 70°C for 48h.

DMF and excess 1-amino-3,3-diethoxypropane were removed at 60-80 °C in vacuo utilizing a rotary evaporator. The remaining crude solid was washed with acetone until no colour was detectable in the washing solution. The product was dissolved in 500 ml water and purified by ultrafiltration utilizing a membrane with a cut-off of 10000 Dalton. When the pH of the retentate had reached a value of 6-7, it was readjusted to 9 utilizing 0.1 M sodium hydroxide solution. This procedure was repeated four times. Finally the product was lyophilised.

Example 2: Preparation of oxHES 55/0.7 Interferon alpha 2b (IFNa) conjugate

[0258] To 400 mg of acetal prepared in example 1 an appropriate amount of 10 mM HCl was added to yield a solution with a concentration of 40 % (w/v). The solution was incubated under stirring at 21 ° C for 24 h to deprotect the aldehyde function. The pH-value was adjusted to the value used in the conjugation buffer by addition of 0.1 M NaOH. Interferon-alpha (recombinant human interferon alpha-2b manufactured by recombinant DNA technology using Escherichia coli (E. coli), the interferon alpha-2b being composed of 165 amino acids and presenting an amino acid sequence which is identical to natural human interferon alpha-2b (hIFN-alpha-2b)) was concentrated up to 16 mg/ml and transferred into a suitable conjugation buffer (0.1 M sodium acetate buffer, pH 4.0) using ultrafiltration devices.

A 10fold molar excess of oxHES aldehyde (based on M_w) was used with a protein concentration in the reaction mixture of 6 mg/ml; the oxHES aldehyde concentration was 20 % (w/v). The deprotected oxHES aldehyde was combined with the protein solution and the reductive amination reaction was started by addition of a freshly prepared $NaCNBH_3$ solution (0.5 M in conjugation buffer) to yield a final concentration of reducing agent of 20 mM. After thorough mixing, the reaction was incubated o/n at 10 °C.

[0259] Reaction mixtures were analyzed by SDS-PAGE (Fig. 1) and reversed phase chromatography on a C 18 column (Phenomenex, Jupiter) to prove successful coupling and for determination of the conjugation yield. Elution was carried out using an acidic water/acetonitrile gradient with 0.1 % TFA.

The HESylated interferon-alpha was separated from non-reacted compounds by anion-exchange chromatography using a Q HP column on an Äkta system (GE Healthcare). Eluent A was 10 mM Tris·Cl, pH 8.0, Eluent B was 10 mM Tris·Cl, 0.5 M NaCl, pH 8.0. The gradient for separation of conjugate and non-modified protein was 0% B => 50% B in 16 CV (Fig. 2).

Example 3: Preparation of oxHES 55/0.7 Erythropoietin (EPO) conjugates

[0260]   To 400 mg of acetal prepared in example 1 an appropriate amount of 10 mM HCl was added to yield a solution with a concentration of 40 % (w/v). The solution was incubated under stirring at 21 °C for 24 h to deprotect the aldehyde function. The pH value was adjusted to the value used in the conjugation buffer by addition of 0.1 M NaOH.
The deprotected oxHES aldehyde was combined with an EPO (recombinant human EPO having the amino acid sequence of human EPO and essentially the same characteristics as the commercially available Erypo® (Ortho Biotech, Jansen-Cilag) or NeoRecormon® (Roche)) solution (10 mg/ml in the reaction buffer 0.1 M sodium acetate buffer, pH 5). OxHES aldehyde was added at a 10fold molar excess (based on M_w) compared to the EPO concentration. The resulting EPO concentration in the reaction mixture was 5 mg/ml, the oxHES aldehyde concentration was 10 % (w/v). The reductive amination reaction was started by addition of a 0.5 M $NaCNBH_3$ solution made up in reaction buffer to yield a final concentration of reducing agent of 20 mM. After thorough mixing, the reaction was incubated o/n at 0 °C.
Reaction mixtures were analyzed by SDS-PAGE (Fig. 3) and reversed phase chromatography on a C 18 column (Phenomenex, Jupiter) to prove successful coupling and for determination of the conjugation yield. Elution of the RP-HPLC was carried out using an acidic water/acetonitrile gradient with 0.1 % TFA.
The HESylated EPO was separated from non-reacted compounds by cation-exchange chromatography using an SP HP column on an Äkta system (GE Healthcare). Eluent A was 20 mM sodium acetate, pH 4.0, Eluent B was 20 mM sodium acetate, 1 M NaCl, pH 4.0. The gradient for separation of conjugate and non-modified protein was 10%B, 2CV; 10% B => 52% B in 21 CV (Fig. 4).
HES coupling sites in the target protein were identified by peptide mapping of the IEX-purified HES-protein conjugate. The conjugates were digested using a suitable protease (2 % Endoproteinase Lys-C, pH 8.6, 37°C, o/n) and the resulting fragments were separated by reversed phase chromatography on a C4 column (Phenomenex, Jupiter) using an acidic water/acetonitrile gradient with TFA. HESylation sites in the protein could be identified indirectly by reduction or disappearance of the respective peptides in the chromatogram as compared to control digests of the target protein alone.

Example 4: Preparation of oxHES100/1.0-N-(3-Propioaldehydediethylacetal)

[0261]   HES aldonic acid (oxHES) was synthesized as described in example 9 of WO 2005/083103 A (in said document, the preparation is described for a hyperbranched starch, HBS) starting from HES with a molecular weight of 100 kDa and a molar substitution of 1.0 (HES100/1.0).
30g oxHES 100/1.0, dried for 2d at 80°C, were dissolved in 150 ml dry dimethylformamide (DMF) and the solution was heated to 70°C. 25g 1-amino-3,3-diethoxypropane in 60 ml dry DMF were added and the reaction mixture was heated at 70°C for 48h.
DMF and excess 1-amino-3,3-diethoxypropane were removed at 60-80 °C in vacuo utilizing a rotary evaporator. The remaining crude solid was washed with acetone until no colour was detectable in the washing solution. The product was dissolved in 500 ml water and purified by ultrafiltration utilizing a membrane with a cut-off of 10,000 Dalton. When the pH of the retentate had reached a value of 6-7, it was readjusted to 9 utilizing 0.1 M sodium hydroxide solution. This procedure was repeated four times. Finally the product was lyophilised.

Example 5: Preparation of HES 100/1.0 Interferon alpha (IFNa) conjugate from oxidized HES

[0262]   To 400 mg of acetal prepared in example 4 an appropriate amount of 10 mM HCl was added to yield a solution with a concentration of 40 % (w/v). The solution was incubated under stirring at 21°C o/n to deprotect the aldehyde function. The pH-value was adjusted to the value used in the conjugation buffer by addition of 0.1 M NaOH.
Interferon-alpha (recombinant human interferon alpha-2b manufactured by recombinant DNA technology using Escherichia coli (E. coli), the interferon alpha-2b being composed of 165 amino acids and presenting an amino acid sequence which is identical to natural human interferon alpha-2b (hIFN-alpha-2b)) was concentrated up to 16 mg/ml and transferred into a suitable conjugation buffer (0.1 M sodium acetate buffer, pH 4.0) using ultrafiltration devices.
[0263]   A 6fold molar excess of oxHES aldehyde (based on M_n) was used with a final protein concentration in the reaction mixture of 8 mg/ml; the oxHES aldehyde concentration was 20 % (w/v). The deprotected oxHES aldehyde was combined with the protein solution and the reductive amination reaction was started by addition of a freshly prepared $NaCNBH_3$ solution (0.5 M in conjugation buffer) to yield a final concentration of reducing agent of 20 mM. After thorough mixing, the reaction was incubated o/n at 5 °C.
Reaction mixtures were analyzed by SDS-PAGE (Fig. 5) and reversed phase chromatography on a C18 column (Phenomenex, Jupiter) to prove successful coupling and for determination of the conjugation yield. Elution was carried out using an acidic water/acetonitrile gradient with 0.1 % TFA.
The HESylated Interferon-alpha was separated from non-reacted compounds by anion-exchange chromatography using a Q HP column on an Äkta system (GE Healthcare). Eluent A was 10 mM Tris·Cl, pH 8.0, Eluent B was 10 mM Tris·Cl,

0.5 M NaCl, pH 8.0. The gradient for separation of conjugate and non-modified protein was 0% B => 50% B in 12.5 CV (Fig. 6).

HES coupling sites in the target protein were identified by peptide mapping of the IEX-purified HES-protein conjugate. The conjugates were digested using a suitable protease (2 % Endoproteinase Lys-C, pH 8.6, 37°C, o/n) and the resulting fragments were separated by reversed phase chromatography on a C4 column (Phenomenex, Jupiter) using an acidic water/acetonitrile gradient with TFA. HESylation sites in the protein could be identified indirectly by reduction or disappearance of the respective peptides in the chromatogram as compared to control digests of the target protein alone (Fig. 7).

Example 6: Preparation of an HES 100/1.0 Erythropoietin (EPO) conjugate from oxidized HES

[0264]   To 400 mg of acetal prepared in example 4 an appropriate amount of 10 mM HCl was added to yield a solution with a concentration of 40 % (w/v). The solution was incubated at 21° C for 24 h to deprotect the aldehyde function. The pH-value was adjusted to the value used in the conjugation buffer by addition of 0.1 M NaOH.

The deprotected oxHES aldehyde was combined with an EPO (recombinant human EPO having the amino acid sequence of human EPO and essentially the same characteristics as the commercially available Erypo® (Ortho Biotech, Jansen-Cilag) or NeoRecormon® (Roche)) solution (10 mg/ml in the reaction buffer 0.1 M sodium acetate buffer, pH 5). OxHES aldehyde was added at a 15fold molar excess (based on M_n) compared to the EPO concentration. The resulting EPO concentration in the reaction mixture was 3.7 mg/ml, the oxHES aldehyde concentration was 15 % (w/v). The reductive amination reaction was started by addition of a 0.5 M NaCNBH$_3$ solution made up in reaction buffer to yield a final concentration of reducing agent of 20 mM. After thorough mixing, the reaction was incubated o/n at 10 °C.

Reaction mixtures were analyzed by SDS-PAGE (Fig. 8) and reversed phase chromatography on a C18 column (Phenomenex, Jupiter) to prove successful coupling and for determination of the conjugation yield. Elution was carried out using an acidic water/acetonitrile gradient with 0.1 % TFA.

The HESylated EPO was separated from non-reacted compounds by cation-exchange chromatography using an SP HP column on an Äkta system (GE Healthcare). Eluent A was 20 mM sodium acetate, pH 4.0, Eluent B was 20 mM sodium acetate, 1 M NaCl, pH 4.0. The gradient for separation of conjugate and non-modified protein was 10 %B, 2 CV; 10% B => 52% B in 21 CV (Fig. 9).

HES coupling sites in the target protein were identified by peptide mapping of the IEX-purified HES-protein conjugate. The conjugates were digested using a suitable protease (2 % Endoproteinase Lys-C, pH 8.6, 37°C, o/n) and the resulting fragments were separated by reversed phase chromatography on a C4 column (Phenomenex, Jupiter) using an acidic water/acetonitrile gradient with TFA. HESylation sites in the protein could be identified indirectly by reduction or disappearance of the respective peptides in the chromatogram as compared to control digests of the target protein alone.

Example 7: Preparation of an HES 100/1.0 Granulocyte colony stimulating factor (G-CSF) conjugate from oxidized HES

[0265]   To 400 mg of acetal prepared in example 4 an appropriate amount of 10 mM HCl was added to yield a solution with a concentration of 40 % (w/v). The solution was incubated at 21 ° C o/n to deprotect the aldehyde function. The pH-value was adjusted to the value used in the conjugation buffer by addition of 0.1 M NaOH.

The deprotected oxHES aldehyde was combined with a rh-Met-G-CSF solution (5 mg/ml in the reaction buffer 0.1 M sodium acetate buffer, pH 5; G-CSF expressed by E.coli having the same amino acid sequence and essentially the same characteristics as the commercially available Neupogen® from Amgen, München, D). OxHES aldehyde was added at a 30fold molar excess (based on M_n) compared to the G-CSF concentration. The resulting G-CSF concentration in the reaction mixture was 1.9 mg/ml, the oxHES aldehyde concentration was 20 % (w/v). The reductive amination reaction was started by addition of a 0.5 M NaCNBH$_3$ solution made up in reaction buffer to yield a final concentration of reducing agent of 20 mM. After thorough mixing, the reaction was incubated o/n at 0 °C. Reaction mixtures were analyzed by SDS-PAGE (Fig. 10) and reversed phase chromatography (Fig. 11) on a C18 column (Phenomenex, Jupiter) to prove successful coupling and for determination of the conjugation yield. Elution was carried out using an acidic water/acetonitrile gradient with 0.1 % TFA.

Example 8: Preparation of HES100/1.0-N-(3-Propioaldehydediethylacetal)

[0266]   15 g HES 100/1.0 were dissolved in 35 g sodium acetate buffer (pH = 5 and c = 1 mol/l) and 2.07 ml of 1-amino-3,3-diethoxypropane as well as 1.885 g of sodium cyano borohydride were added. The reaction mixture was stirred at 60 °C for 16-24 h, diluted with 100 ml water, neutralized with diluted sodium hydroxide solution and worked up by ultrafiltration using a membrane with a cut-off of 10,000 Da against ammonium hydrogen carbonate buffer (pH = 9, c = 10 mmol/l, 45 cycles) as well as water for the last 5 exchange cycles. The purified and concentrated HES derivative solution (approximately 20 wt-%) was dialyzed against sodium hydroxide solution (pH = 12) at 60 °C using a membrane

with a cut off of 10,000 Da. Thereafter the product was isolated by lyophilisation.

Example 9: Preparation of HES100/1.0-N-(3-Propioaldehyde)

[0267] 10 g of HES-N-(3-Propioaldehydediethylacetal) from example 8 were dissolved in 100 ml of aqueous HCl, pH = 2 (c = 10 mmol/l) and stirred at 40 °C for 16-24 h. The reaction mixture was purified by ultrafiltration using a membrane with a cut off of 10,000 Da against aqueous HCl, pH = 2 (10 cycles) as well as water for the last 5 exchange cycles. The isolation of the product was carried out by lyophilisation.

Example 10: Preparation of an HES 100/1.0 Interferon alpha (IFNa) conjugate from HES

[0268] To 400 mg of acetal prepared in example 8 an appropriate amount of 10 mM HCl was added to yield a solution with a concentration of 40 % (w/v). The solution was incubated under stirring at 21 ° C o/n to deprotect the aldehyde function. The pH-value was adjusted to the value used in the conjugation buffer by addition of 0.1 M NaOH.
Interferon-alpha (recombinant human interferon alpha-2b manufactured by recombinant DNA technology using Escherichia coli (E. coli), the interferon alpha-2b being composed of 165 amino acids and presenting an amino acid sequence which is identical to natural human interferon alpha-2b (hIFN-alpha-2b)) was concentrated up to 16 mg/ml and transferred into a suitable conjugation buffer (0.1 M sodium acetate buffer, pH 4.0) using ultrafiltration devices.
[0269] A 5fold molar excess of HES aldehyde (based on M_w) was used with a final protein concentration in the reaction mixture of 7 mg/ml; the HES aldehyde concentration was 18 % (w/v). The deprotected HES aldehyde was combined with the protein solution and the reductive amination reaction was started by addition of a freshly prepared $NaCNBH_3$ solution (0.5 M in conjugation buffer) to yield a final concentration of reducing agent of 20 mM. After thorough mixing, the reaction was incubated o/n at 5 °C.
Reaction mixtures were analyzed by SDS-PAGE (Fig. 12) and reversed phase chromatography on a C 18 column (Phenomenex, Jupiter) to prove successful coupling and for determination of the conjugation yield. Elution was carried out using an acidic water/acetonitrile gradient with 0.1 % TFA.
The HESylated IFNalpha was separated from non-reacted compounds by cation-exchange chromatography using an SP HP column on an Äkta system (GE Healthcare). Eluent A was 20 mM sodium acetate, pH 4.0, Eluent B was 20 mM sodium acetate, 1 M NaCl, pH 4.0. The gradient for separation of conjugate and non-modified protein was 0% B => 50% B in 20 CV (Fig. 13).
HES coupling sites in the target protein were identified by peptide mapping of the IEX-purified HES-protein conjugate. The conjugates were digested using a suitable protease (2 % Endoproteinase Lys-C, pH 8.6, 37°C, o/n) and the resulting fragments were separated by reversed phase chromatography on a C4 column (Phenomenex, Jupiter) using an acidic water/acetonitrile gradient with TFA. HESylation sites in the protein could be identified indirectly by reduction or disappearance of the respective peptides in the chromatogram as compared to control digests of the target protein alone.

Example 11: Preparation of an HES 100/1.0 Erythropoietin (EPO) conjugate from HES

[0270] The deprotected HES aldehyde from example 9 was combined with an EPO (recombinant human EPO having the amino acid sequence of human EPO and essentially the same characteristics as the commercially available Erypo® (Ortho Biotech, Jansen-Cilag) or NeoRecormon® (Roche)) solution (10 mg/ml in the reaction buffer 0.1 M sodium acetate buffer, pH 5). HES aldehyde was added at a 40fold molar excess (based on M_n) compared to the EPO concentration. The resulting EPO concentration in the reaction mix was 3.2 mg/ml, the HES aldehyde concentration was 30 % (w/v). The reductive amination reaction was started by addition of a 0.5 M $NaCNBH_3$ solution made up in reaction buffer to yield a final concentration of reducing agent of 20 mM. After thorough mixing, the reaction was incubated o/n at 5 °C.
Reaction mixtures were analyzed by SDS-PAGE (Fig. 14) and reversed phase chromatography on a C 18 column (Phenomenex, Jupiter) to prove successful coupling and for determination of the conjugation yield. Elution was carried out using an acidic water/acetonitrile gradient with 0.1% TFA.
The HESylated EPO was separated from non-reacted compounds by cation-exchange chromatography using an SP HP column on an Äkta system (GE Healthcare). Eluent A was 20 mM sodium acetate, pH 4.0, Eluent B was 20 mM sodium acetate, 1 M NaCl, pH 4.0. The gradient for separation of conjugate and non-modified protein was 0% B => 52% B in 13 CV (Fig. 15).

Example 12: Preparation of an HES 100/1.0 Granulocyte Colony stimulating Factor (G-CSF) conjugate from HES

[0271] The deprotected HES aldehyde from example 9 was combined with a rh-Met-G-CSF solution (5 mg/ml in the reaction buffer 0.1 M sodium acetate buffer, pH 5; G-CSF expressed by E.coli having the same amino acid sequence and essentially the same characteristics as the commercially available Neupogen® from Amgen, München, D). HES

aldehyde was added at a 40fold molar excess (based on M_n) compared to the G-CSF concentration. The resulting G-CSF concentration in the reaction mix was 1.3 mg/ml, the HES aldehyde concentration was 20 % (w/v). The reductive amination reaction was started by addition of a 0.5 M $NaCNBH_3$ solution made up in reaction buffer to yield a final concentration of reducing agent of 20 mM. After thorough mixing, the reaction was incubated o/n at 10 °C.

Reaction mixtures were analyzed by SDS-PAGE and reversed phase chromatography on a C18 column (Phenomenex, Jupiter) to determine the conjugation yield. Elution of the RP-HPLC was carried out using an acidic water/acetonitrile gradient with 0.1 % TFA. Reaction mixtures were analyzed by SDS-PAGE (Fig. 16) and reversed phase chromatography (Fig. 17) on a C18 column (Phenomenex, Jupiter) to prove successful coupling and for determination of the conjugation yield. Elution of the RP-HPLC was carried out using an acidic water/acetonitrile gradient with 0.1 % TFA.

The HESylated G-CSF was separated from non-reacted compounds by cation-exchange chromatography using an SP HP column on an Äkta system (GE Healthcare). Eluent A was 20 mM sodium acetate, pH 4.0, Eluent B was 20 mM sodium acetate, 1 M NaCl, pH 4.0. The gradient for separation of conjugate and non-modified protein was 0% B => 40% B in 15 CV (Fig. 18).

Example 13: Pharmacodynamic in-vivo Bioassay in Mice (HES-EPO conjugate according to Example 11)

[0272]   Balb C mice obtained from Harlan Winkelmann GmbH (Borchen, Germany) weighing approximately 18-20 grams were group housed (max. 10 per cage) in Euro Standard Typ III (LxBxH 425x266x185mm) cages at a room temperature of 21 °C and a relative humidity of 55%. "Tapvei Einstreu" 4x4xlmm (wood of Aspen) was used as bedding material for the cages. Additionally wood wool was offered. The cages were changed and cleaned once a week. Drinking water (pH 3.8-4; sulfuric acid) was offered ad libitum. The animal cages were numbered. Within a cage the animals were ear marked and additionally colour coded. On the day of allocation approx. and one week before commencement of treatment, an initial health check has been performed. Only healthy animals were used.

The HES EPO conjugate as obtained in example 11, the unmodified starting material (rHuEPO) and Aranesp® from Amgen were tested as a single bolus, subcutaneous dose in 4 mice per group at a dosage of 100 µg/kg body weight, based on the protein content of the samples. The same volume of PBS as vehicle control was included.

At several time points (day 0, 3, 6, 9, 13, 16, 20, and 23) samples of approximately 30-60 µl whole blood were taken from the tail vein or the retrobulbar venous plexus using "Hämatokrit-Kapillaren" containing Na-heparin (Hirschmann Laborgeräte, Germany) and the whole blood was centrifuged for 6 minutes at 10.000 rpm in a Hettich Hämatocrit 210 centrifuge (Tuttlingen, Germany) to determine the hematocrit of each whole blood sample.

The erythropoietic response and duration were monitored as a function change of hematocrit [%] as a function of time (see Fig. 19)

These data show that all samples containing EPO, Aranesp® or EPO conjugate were capable in raising the hematocrit. Aranesp® was capable to increase the potency compared to starting material 3-4 fold and as well the HES EPO conjugate was capable to increase the potency of 1.5 - 2 fold compared to Aranesp®.

Example 14: Pharmacodynamic in-vivo Bioassay in Mice (HES-IFN alpha conjugate according to Example 5)

[0273]   One oxHES100/1.0 Interferon alpha Conjugate, prepared according to Example 5 was tested in the in vivo assay according to example 13. The EC50 dilution of the serum samples was half-logarithmically plotted against the time after iv.-injection. Half-life was calculated from the slope of the exponential fit-curve. The half life of the sample was 8.9 hours (see Figure 20).

[0274]   The relative *in vitro* activity of an oxHES100/1.0 Interferon alpha conjugate, prepared according to Example 5 compared to Intron A is shown in Fig. 21 (as to the determination of the *in vitro* activity, see example 16 below).

Example 15: Pharmacodynamic in-vivo Bioassay in Mice (HES-IFN alpha conjugate according to Example 10)

[0275]   Three HES100/1.0 Interferon alpha Conjugates, prepared according to Example 10 were tested in the in vivo assay according to example 13. The medium of the EC50 dilution of the serum samples was half-logarithmically plotted against the time after iv.-injection. Half-life was calculated from the slope of the exponential fit-curve. The average half life of the samples was 9.7 hours (Figure 22).

For unmodified IFN-alpha, the antiviral activity of serum was too low to calculate a serum half-life. In K.R. Reddy et al. Advaced Drug Delivery Reviews 54 (2002) pp. 571-586 a serum half-life of IFN-alpha in rats (i.v.) of 2 h was determined.

[0276]   The relative *in vitro* activity of three HES100/1.0 Interferon alpha Conjugates, prepared according to Example 10 compared to Intron A is shown in Fig. 23 (as to the determination of the *in vitro* activity, see example 16 below).

Example 16: Description of the Test Procedure: Antiviral activity of Interferon-alpha (examples 14 and 15)

[0277]   After pre-diluting the Test Items in cell culture medium, serial two-fold dilutions were prepared. In 96 well microtiter plates, diluted Interferon was added - in four-fold replicate per dilution- to freshly trypsinized MDBK cells (40.000 cells per well). The assays were incubated for 24 hours at 37 °C (total volume per well: 175 μl.

Subsequently, 50 μL diluted VSV stock solution were added to each well (except for the positive control wells) resulting in a multiplicity of infection of 0.1.

The following controls were included in each assay: 12 wells that received virus plus cell culture medium instead of Interferon (negative control) and 12 wells that received cell culture medium instead of Interferon and virus (positive control).

The assays were incubated for 42 hours at 37 °C.

At the end of the incubation period the cell culture supernatant of each well was replaced with 50 μL of a solution of MTT (at least 2 mg/mL in cell culture medium). The cells were incubated for three hours. The purple formazan dye formed by the proliferating cells was solubilized by adding 100 μL solution of isopropanol/HCl (isopropanol with 40 mM HCl) to each well. Subsequently, the absorbance values of the solutions were measured at 570/630 nm in a microtiter plate reader.

The proliferative activity of MDBK cells grown in the presence of Interferon and VSV was calculated for each dilution of Interferon as follows:

$$\frac{((\text{Mean absorbance of four Interferon treated wells}) - (\text{Mean absorbance of negative control})) * 100}{(\text{Mean absorbance of positive control}) - (\text{Mean absorbance of negative control})}$$

[0278]   The antiviral activity of Interferon-alpha was determined in four separate assays for each of the Test Items.

[0279]   In the assay system described above, the respective conjugates HES100/1.0 Interferon alpha Conjugate (example 15 and 10, respectively) and oxHES100/1.0-Interferon alpha Conjugate (example 14 and 5, respectively) were tested compared to unmodified IFN-alpha starting material, namely Intron A. The CPE50 concentration of the materials was calculated..

Additional Data

(A.1) Preparation of oxHBS-N-(3-Propioaldehydediethylacetal) from HBS 7kDa

[0280]   Hyper branched starch (HBS) aldonic acid was synthesized according to example 9 of WO2005 / 083103 A1 starting from a hyperbranched starch (M_w=7000 Dalton (7kDa), average degree of branching: 15 mol %). The aldonic acid obtained was transferred into the corresponding lactone by drying for 24 h at 80 °C (the abbreviation "oxHBS" refers to the HBS aldonic acid as well as to the corresponding lactone).

5 g of the lactone were dissolved in 15 ml 1-amino-3,3-diethoxypropane and 10 ml of dry DMF and stirred at 70 °C for 48 h. Excess 1-amino-3,3-diethoxypropane and DMF (dimethylformamide) were evaporated under vacuum and the resulting pale yellow solid was washed with acetone until the yellow colour disappeared. The product was dissolved in water and purified by ultrafiltration utilizing a membrane with a cut-off of 1000 Dalton until the pH of the filtrate reached a value of> 6.

The retentate was treated with 2 g of an acidic cation exchange resign (Amberlite® 120) for 2 h, the resign was filtered off and the remaining solution lyophilized.

The ^{1}H-NMR Spectrum of the compound showed a triplett at 1.7 and a multiplett at 1.2 ppm representing the methyl- and the methylene groups in alpha-position to the nitrogen atom of the residue of the linker compound (1-amino-3,3-diethoxypropane).

(A.2) Preparation of oxHBS 7kDa - Bovine Serum Albumin (BSA) conjugate

[0281]   750 μg of acetal prepared in (A.1) were dissolved in 5 ml 0.01 N HCl. The pH was adjusted to 2.0 with 1 N HCl, and the reaction mixture was stirred at 21 °C for 18 h. 2 ml of a 1 % BSA solution in acetate buffer (pH = 7.0) were added to 200 μl of the mixture prepared before. 140 mg sodium cyanoborohydride were dissolved in 5 ml 0.1 N acetate buffer (pH = 7.0), and an aliquot of 50 ml was added to the reaction mixture immediately. The reaction mixture was stored at 4 °C for 15 h. Analysis of the reaction mixture by size-exclusion chromatography revealed a reaction yield of 90 % HBS-BSA conjugate. (Figure 24)

(A.3) Preparation of oxHBS 65kDa - Interferon-alpha conjugate

[0282]   To 400 mg of a 65kDa HBS-N-(3-propioaldehydediethylacetal) prepared analogously to (A.1) an appropriate amount of 10 mM HCl was added to yield a solution with a concentration of 40 % (w/v) and a pH value of 2. The solution was incubated under stirring at 21°C o/n (overnight) to deprotect the aldehyde function. The pH-value was adjusted to the value used in the conjugation buffer by addition of 0.1 M NaOH prior to coupling. Interferon-alpha (recombinant human interferon alpha-2b manufactured by recombinant DNA technology using Escherichia coli (E. coli), the interferon alpha-2b being composed of 165 amino acids and presenting an amino acid sequence which is identical to natural human interferon alpha-2b (hIFN-alpha-2b)) was concentrated up to 16 mg/ml and transferred into a suitable conjugation buffer (0.1 M sodium acetate buffer, pH 4.0) using ultrafiltration devices.

A 10fold molar excess of oxHBS aldehyde (based on M_w) was used with a final protein concentration in the reaction mixture of 6 mg/ml; the oxHBS aldehyde concentration was 20 % (w/v). The deprotected oxHBS aldehyde was combined with the protein solution and the reductive amination reaction was started by addition of a freshly prepared $NaCNBH_3$ solution (0.5 M in conjugation buffer) to yield a final concentration of reducing agent of 20 mM. After thorough mixing, the reaction was incubated o/n at 10 °C.

The reaction mixtures were analyzed by SDS-PAGE (Fig. 25) and reversed phase chromatography on a C 18 column (Phenomenex, Jupiter) to prove successful coupling and for determination of the conjugation yield. Elution was carried out using an acidic water/acetonitrile gradient with 0.1 % TFA.

The HBS-Interferon-alpha was separated from non-reacted compounds by anion-exchange chromatography using a Q HP column on an Äkta system (GE Healthcare). Eluent A was 10 mM Tris·Cl, pH 8.0, Eluent B was 10 mM Tris·Cl, 0.5 M NaCl, pH 8.0. The gradient for separation of conjugate and non-modified protein was 0% B => 50% B in 16 CV (Fig. 26).

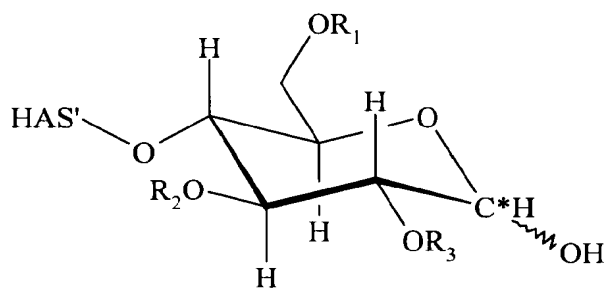## (A.4) Preparation of oxHBS 65 Erythropoietin (EPO) conjugate

[0283]   To 400 mg of a 65kDa HBS-N-(3-propioaldehydediethylacetal) prepared analogously to (A.1) an appropriate amount of 10 mM HCl was added to yield a solution with a concentration of 40 % (w/v). The solution was incubated under stirring at 21 ° C o/n to deprotect the aldehyde function. The pH-value was adjusted to the value used in the conjugation buffer by addition of 0.1 M NaOH.

The deprotected oxHBS aldehyde was combined with an EPO (recombinant human EPO having the amino acid sequence of human EPO and essentially the same characteristics as the commercially available Erypo® (Ortho Biotech, Jansen-Cilag) or NeoRecormon® (Roche)) solution (10 mg/ml in the reaction buffer 0.1 M sodium acetate buffer, pH 5). OxHBS aldehyde was added at a 20fold molar excess (based on M_w) compared to the EPO concentration. The resulting EPO concentration in the reaction mix was 4.6 mg/ml, the oxHBS aldehyde concentration was 20 % (w/v). The reductive amination reaction was started by addition of a 0.5 M $NaCNBH_3$ solution made up in reaction buffer to yield a final concentration of reducing agent of 20 mM. After thorough mixing, the reaction was incubated o/n at 10 °C.

The reaction mixtures were analyzed by SDS-PAGE (Fig. 27) and reversed phase chromatography (Fig. A.4-1) on a C18 column (Phenomenex, Jupiter) to prove successful coupling and for determination of the conjugation yield. Elution was carried out using an acidic water/acetonitrile gradient with 0.1 % TFA.

Claims

1. A method for the preparation of a hydroxyalkyl starch derivative, comprising (i) reacting hydroxyalkyl starch (HAS) of formula (I)
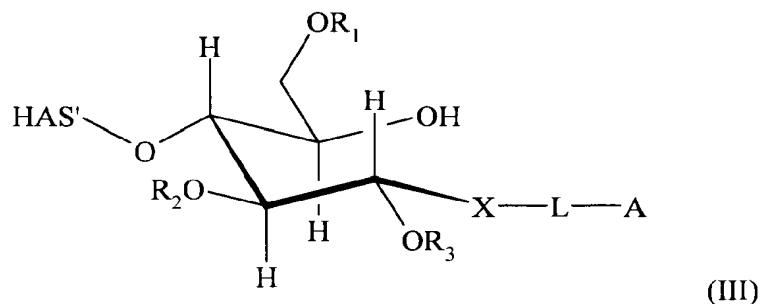

(I)

via carbon atom C* of the reducing end of the HAS with the amino group M of a crosslinking compound according to formula (II)

M-L-A   (II)

wherein A is an acetal group or a ketal group; and L is a spacer bridging M and A, wherein C* is optionally oxidised prior to the reaction of HAS with M, obtaining a HAS derivative according to formula (III)
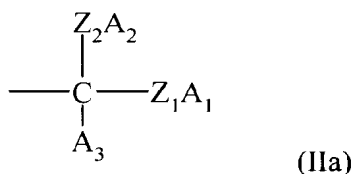

(III)

wherein X is the functional group resulting from the reaction of the amino group M with the HAS via carbon atom C* of the optionally oxidised reducing end of the HAS, and
wherein HAS' is the remainder of the hydroxyalkyl starch molecule, and R_1, R_2 and R_3 are independently hydrogen or a linear or branched hydroxyalkyl group.

2. The method of claim 1, wherein R_1, R_2 and R_3 are independently a group $-(CH_2CH_2O)_n-H$, wherein n is an integer, preferably 0, 1, 2, 3, 4, 5, or 6.

3. The method of claim 1 or 2, wherein the hydroxyalkyl starch is hydroxyethyl starch (HES).

4. The method of any of claims 1 to 3, wherein A is a residue according to formula (IIa)

(IIa)

wherein

Z_1 and Z_2 are each independently O or S or NR_x, preferably O, wherein R_x is H or lower alkyl such as methyl, ethyl, or propyl such as n-propyl or i-propyl, or $C(O)-R_y$ wherein R_y is preferably selected from the group consisting of C_1-C_6 alkyl and C_6-C_{14} aryl, even more preferably selected from the group consisting of optionally substituted, preferably non-substituted methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, and tert-butyl; R_x preferably being H;
A_1 and A_2 are each independently methyl, ethyl, n-propyl, isopropyl, n-butyl, tert-butyl, benzyl, 1,1,1-trichloroe-thyl, nitrobenzyl, methoxybenzyl, ethoxybenzyl, or are forming a ring according to formula (IIb)

$$\begin{array}{c} Z_2A_2 \\ | \\ -C-Z_1A_1 \\ | \\ A_3 \end{array}$$

(IIb)

wherein A_1 and A_2, taken together, are $-(CH_2)_2-$ or $-(CH_2)_3-$ or $-(CH_2CH(CH_3))-$, and
wherein A_3 is H or methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, pentyl, benzyl, or is forming a ring with the N atom of the amino group M or with a suitable atom comprised in L, A_3 preferably being H.

5. The method of claim 4, wherein Z_1 and Z_2 are each O; A_3 is H; and wherein A_1 and A_2 are each ethyl or wherein A_1 and A_2 are forming a ring according to formula (IIb) wherein A_1 and A_2, taken together, are $-(-CH_2)_2-$.

6. The method of any of claims 1 to 5, wherein the amino group M is a group according to formula (IIc)
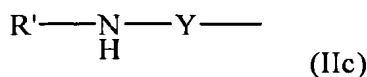
$$R'-\underset{H}{N}-Y-$$

(IIc)

wherein Y is either absent or is a chemical moiety selected from the group consisting of
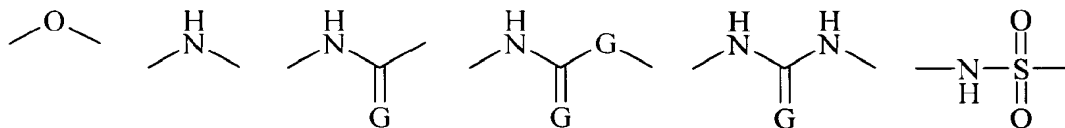
wherein G is O or S or NH, and, if present twice, each G is independently O or S or NH, O being preferred, and wherein R' is H or a hydroxy group or an organic residue selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, alkylaryl, and substituted alkylaryl; preferably H or an organic residue selected from the group consisting of alkyl and substituted alkyl; more preferably H or alkyl.

7. The method of any of claims 1 to 6, wherein the amino group M is H_2N-, H_2N-O-, $H_2N-NH-(C=O)-$, H_3C-NH- or $H_3C-NH-O-$, preferably H_2N-, H_2N-O-, or $H_2N-NH-(C=O)-$.

8. The method of any of claims 1 to 7, wherein in (i), HAS is reacted via its oxidised reducing end with the amino group M of the crosslinking compound, M being H_2N-, and wherein the reaction is carried out at a temperature in the range of from 0 to 80 °C, and wherein X is $-(C=O)-NH-$.

9. The method of any of claims 1 to 7, wherein in (i), HAS is reacted, preferably in an aqueous system, via its non-oxidised reducing end with the amino group M of the crosslinking compound, M being H_2N-, and wherein the reaction is carried out at a temperature in the range of from 20 to 80 °C at a pH in the range of from 4 to 7, X being $-CH=N-$.

10. The method of claim 9, wherein in (i), the reaction is carried out in the presence of a reductive agent, preferably $NaCNBH_3$, to obtain a HAS derivative, X being $-CH_2-NH-$.

11. The method of any of claims 1 to 7, wherein in (i), HAS is reacted, preferably in an aqueous system, via its non-oxidised reducing end with the amino group M of the crosslinking compound, M being H_2N-O- or $H_2N-NH-(C=O)-$, and wherein the reaction is carried out at a temperature in the range of from 5 to 80 °C at a pH in the range of from 4.5 to 6.5, X being $-CH=N-O-$ or $-CH=N-NH-(C=O)-$.

12. The method of any of claims 1 to 11, wherein L bridging M and A is a spacer comprising at least one structural unit according to formula (IId), preferably consisting of a structural unit according to formula (IId)

$$
+\!\!\left(\!\!\begin{array}{c} L_1 \\ | \\ C \\ | \\ L_2 \end{array}\!\!\right)_{\!\!n}\!\!-
$$

(IId)

wherein L_1 and L_2 are independently from each other H or an organic residue selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, alkylaryl, substituted alkylaryl, and residues -O-R'' wherein R'' is selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, alkylaryl, substituted alkylaryl; preferably H or an organic residue selected from the group consisting of alkyl and substituted alkyl; more preferably H or alkyl; more preferably H,
wherein n is an integer from 1 to 20, preferably from 1 to 10, more preferably from 1 to 6, more preferably from 1 to 4, more preferably 2.

13. The method of any of claims 1 to 12, wherein L is $-CH_2-CH_2-$.

14. The method of any of claims 1 to 13, wherein the crosslinking compound M-L-A according to formula (II) is 1-amino-3,3-diethoxypropane.

15. The method of any of claims 1 to 14, further comprising

(ii) reacting the HAS derivative according to formula (III) via group A with an amino group of a biologically active agent H_2N-BA', via reductive amination, obtaining a HAS derivative according to formula (IV)

(IV)
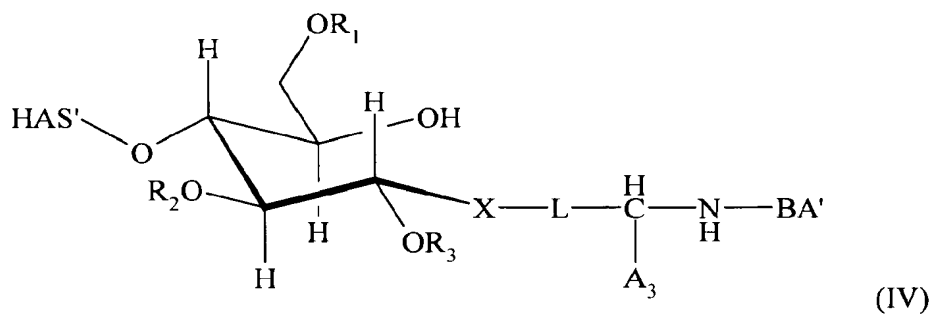
16. The method of claim 15, wherein prior to (ii), group A of the HAS derivative according to formula (III) is transformed to the corresponding aldehyde or keto group.

17. The method of claim 15 or 16, wherein in (ii), the reaction is carried out, preferably in an aqueous system, in the presence of a reducing agent, preferably $NaCNBH_3$, at a temperature in the range of from 0 to 37 °C, preferably 0 to 25 °C and a pH in the range of from 3 to 9, preferably from 3 to 7, more preferably from 3 to below 7, and wherein in (ii), the molar ratio of the HAS derivative to biologically active agent BA is from 0.1:1 to 200:1 equivalents, preferably from 1:1 to 50:1 equivalents, based on the number average molecular weight (M_n) of the HAS derivative.

18. The method of any of claims 15 to 17, wherein the biologically active compound BA is a peptide, an oligopeptide, a polypeptide, a protein, a functional derivative, fragment or mimetic of the polypeptide or protein, a small molecule compound or an oligonucleotide.

19. The method of claim 18, wherein the protein is erythropoietin (EPO) such as recombinant human EPO (rhEPO), a colony-stimulating factor (CSF) such as G-CSF like recombinant human G-CSF (rhG-CSF), interferon (IFN) such as IFN alpha, IFN beta, IFN gamma like recombinant human IFN alpha (rhIFN alpha) or recombinant human IFN beta (rhIFN beta), factor VII, factor IX, interleukine-2, interleukine-11, alpha-1-antitrypsin, an antibody, or an antibody fragment, or an alternative protein scaffold.
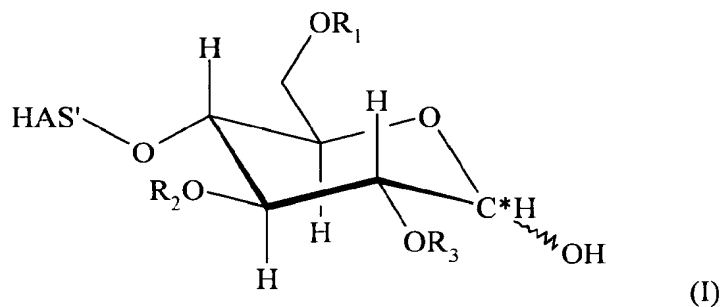
20. A hydroxyalkyl starch (HAS) derivative of formula (III)
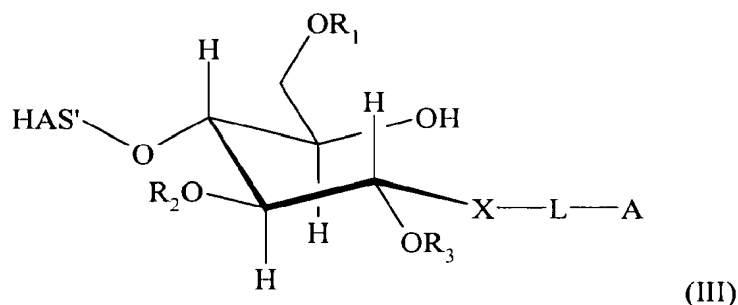
(III)

wherein A is an acetal or ketal group; L is a spacer bridging X and A;
wherein X is the functional group resulting from the reaction of an amino group M of a crosslinking compound of formula (II)

M-L-A   (II)

with hydroxyalkyl starch (HAS) of formula (I)

(I)

via carbon atom C* of the HAS, wherein C* is optionally oxidised prior to the reaction of HAS with M,
wherein HAS' is the remainder of the hydroxyalkyl starch molecule and R_1, R_2 and R_3 are independently hydrogen or a linear or branched hydroxyalkyl group.

21. The HAS derivative of claim 20, wherein R_1, R_2 and R_3 are independently a group - $(CH_2CH_2O)_n$-H, wherein n is an integer, preferably 0, 1, 2, 3, 4, 5, or 6.

22. The HAS derivative of claim 20 or 21, wherein the hydroxyalkyl starch is hydroxyethyl starch (HES).

24. The HAS derivative of any of claims 20 to 22, wherein A is a residue according to formula (IIa)

$$\begin{array}{c} Z_2A_2 \\ | \\ \text{——C——}Z_1A_1 \\ | \\ A_3 \end{array}$$

(IIa)

wherein

Z_1 and Z_2 are each independently O or S or NR_x, preferably O, wherein R_x is H or lower alkyl such as methyl, ethyl, or propyl such as n-propyl or i-propyl, or $C(O)-R_y$ wherein R_y is preferably selected from the group consisting of C_1-C_6 alkyl and C_6-C_{14} aryl, even more preferably selected from the group consisting of optionally substituted, preferably non-substituted methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, and tert-butyl; R_x preferably being H;

A_1 and A_2 are each independently methyl, ethyl, n-propyl, isopropyl, n-butyl, tert-butyl, benzyl, 1,1,1-trichloroethyl, nitrobenzyl, methoxybenzyl, ethoxybenzyl, or are forming a ring according to formula (IIb)

$$\begin{array}{c} Z_2A_2\diagdown \\ | \\ \text{——C——}Z_1A_1 \\ | \\ A_3 \end{array}$$

(IIb)

wherein A_1 and A_2, taken together, are $-(CH_2)_2-$ or $-(CH_2)_3-$ or $-(CH_2CH(CH_3))-$, and
wherein A_3 is H or methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, pentyl, benzyl, or is forming a ring with the N atom of the amino group M or with a suitable atom comprised in L, A_3 preferably being H.

25. The HAS derivative of any of claims 20 to 24, wherein X is selected from the group consisting of -CH=N-, -CH_2-NH-, -CH=N-O-, -CH_2-NH-O-, -C(=O)-NH-, -C(=O)-NH-NH-, preferably consisting of -CH_2-NH-, -CH=N-, -CH=N-O-, -CH_2-NH-O- and -CH=N-NH-(C=O)-.

26. The HAS derivative of any claims 20 to 25, wherein L bridging M and A is a spacer comprising at least one structural unit according to formula (IId), preferably consisting of a structural unit according to formula (IId)

$$\begin{array}{c} L_1 \\ | \\ \text{——}(\text{C})_n\text{——} \\ | \\ L_2 \end{array}$$

(IId)

wherein L_1 and L_2 are independently from each other H or an organic residue selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, alkylaryl, substituted alkylaryl, and residues -O-R'' wherein R'' is selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, alkylaryl, substituted alkylaryl.; preferably H or an organic residue selected from the group consisting of alkyl and substituted alkyl; more preferably H or alkyl; more preferably H,
wherein n is an integer from 1 to 20, preferably from 1 to 10, more preferably from 1 to 6, more preferably from 1 to 4, more preferably 2.
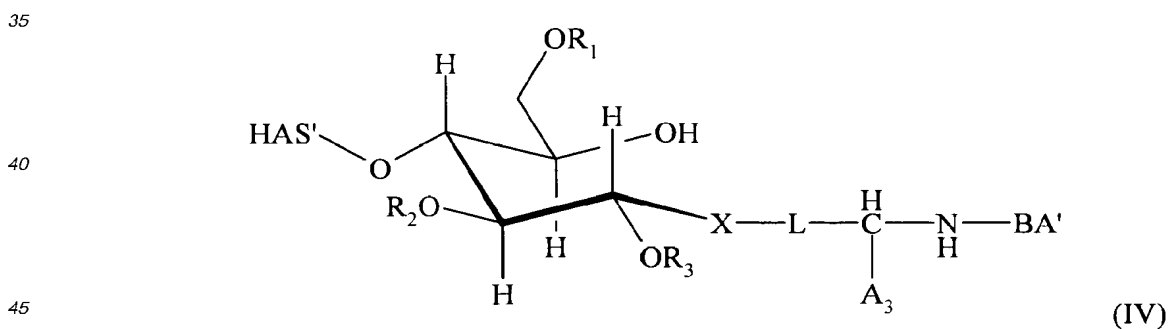
27. The hydroxyalkyl starch derivative of any of claims 20 to 26, having the structure

$$HAS'-O-\text{(sugar ring, OR}_1, H, OH, R_2O, OR_3, H)-C H_2-N H-C H_2-C H_2-CH(OCH_2CH_3)(OCH_2CH_3)$$

28. The hydroxyalkyl starch derivative of any of claims 20 to 26, having the structure

$$HAS'-O-\text{(sugar ring, OR}_1, H, OH, R_2O, OR_3, H)-C(=O)-N H-C H_2-C H_2-CH(OCH_2CH_3)(OCH_2CH_3)$$

29. A hydroxyalkyl starch derivative of formula (IV)

$$HAS'-O-\text{(sugar ring, OR}_1, H, OH, R_2O, OR_3, H)-X-L-\underset{A_3}{\overset{H}{C}}-NH-BA'$$
(IV)

wherein X is a functional group resulting from the reaction of an amino group M of a crosslinking compound of formula (II)

M-L-A   (II)

wherein X is not an amide group -C(=O)-NH-,
with hydroxyalkyl starch (HAS) of formula (I)

(I)

via carbon atom C* of the HAS, wherein C* is optionally oxidised prior to the reaction of HAS with M,
wherein HAS' is the remainder of the hydroxyalkyl starch molecule and R_1, R_2 and R_3 are independently hydrogen or a linear or branched hydroxyalkyl group,
wherein A is a residue according to formula (IIa)

(IIa)

wherein

Z_1 and Z_2 are each independently O or S or NR_x, preferably O, wherein R_x is H or lower alkyl such as methyl, ethyl, or propyl such as n-propyl or i-propyl, or $C(O)-R_y$ wherein R_y is preferably selected from the group consisting of C_1-C_6 alkyl and C_6-C_{14} aryl, even more preferably selected from the group consisting of optionally substituted, preferably non-substituted methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, and tert-butyl; R_x preferably being H;
A_1 and A_2 are each independently methyl, ethyl, n-propyl, isopropyl, n-butyl, tert-butyl, benzyl, 1,1,1-trichloroethyl, nitrobenzyl, methoxybenzyl, ethoxybenzyl, or are forming a ring according to formula (IIb)

(IIb)

wherein A_1 and A_2, taken together, are $-(CH_2)_2-$ or $-(CH_2)_3-$ or $-(CH_2CH(CH_3))-$, and
wherein A_3 is H or methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, pentyl, benzyl, or is forming a ring with the N atom of the amino group M or with a suitable atom comprised in L, A_3 preferably being H.;
and wherein L is a spacer bridging M and A,
wherein BA' is the remainder of a biologically active agent $BA'-NH_2$ remaining after the reaction of the amino group of BA via reductive amination with A or with the aldehyde group or keto group corresponding to A.

30. The HAS derivative of claim 29, wherein X is selected from the group consisting of $-CH_2-NH-$, $-CH=N-$, $-CH_2-NH-O-$, and $-CH=N-O-$, most preferably $-CH_2-NH-$ and $-CH_2-NH-O-$, most preferably $-CH_2-NH-$.

31. The HAS derivative of claim 29 or 30, wherein L bridging M and A is a spacer comprising at least one structural

unit according to formula (IId), preferably consisting of a structural unit according to formula (IId)

$$
\left(\begin{array}{c} L_1 \\ | \\ -C- \\ | \\ L_2 \end{array} \right)_n \qquad \text{(IId)}
$$

wherein L_1 and L_2 are independently from each other H or an organic residue selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, alkylaryl, substituted alkylaryl, and residues -O-R" wherein R" is selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, alkylaryl, substituted alkylaryl; preferably H or an organic residue selected from the group consisting of alkyl and substituted alkyl; more preferably H or alkyl; more preferably H,

wherein n is an integer from 1 to 20, preferably from 1 to 10, more preferably from 1 to 6, more preferably from 1 to 4, more preferably 2.

32. The HAS derivative of any of claims 29 to 31, having the structure
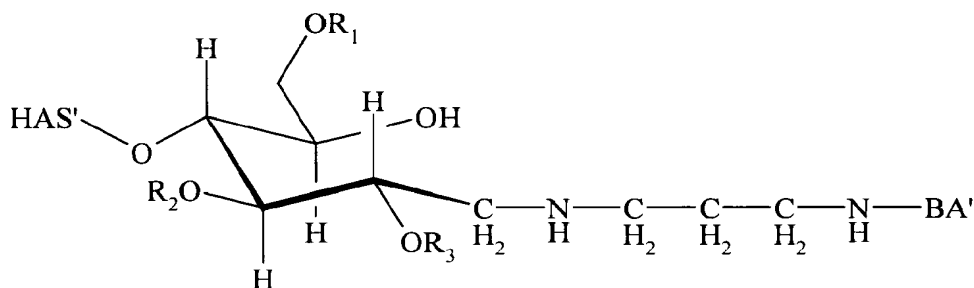
33. The HAS derivative of any of claims 29 to 32, wherein the biologically active compound BA is a peptide, an oligopeptide, a polypeptide, a protein, a functional derivative, fragment or mimetic of the polypeptide or protein, a small molecule compound or an oligonucleotide.

34. The HAS derivative of claim 33, wherein the protein is erythropoietin (EPO) such as recombinant human EPO (rhEPO), a colony-stimulating factor (CSF) such as G-CSF like recombinant human G-CSF (rhG-CSF), interferon (IFN) such as IFN alpha, IFN beta, IFN gamma like recombinant human IFN alpha (rhIFN alpha) or recombinant human IFN beta (rhIFN beta), factor VII, factor IX, interleukine-2, interleukine-11, alpha-1-antitrypsin, an antibody, or an antibody fragment, or an alternative protein scaffold.

35. A HAS derivative according to any of claims 29 to 34 as a therapeutic or prophylactic agent.

36. A HAS derivative according to any of claims 29 to 34 for use in a method for the treatment of the human or animal body.

37. A pharmaceutical composition comprising, in a therapeutically effective amount, a HAS derivative according to any of claims 29 to 34.
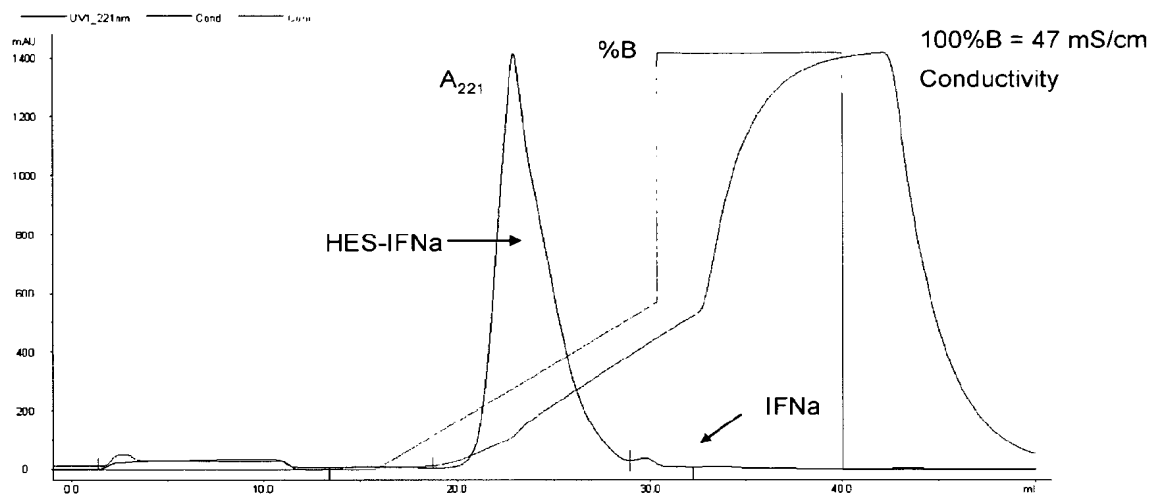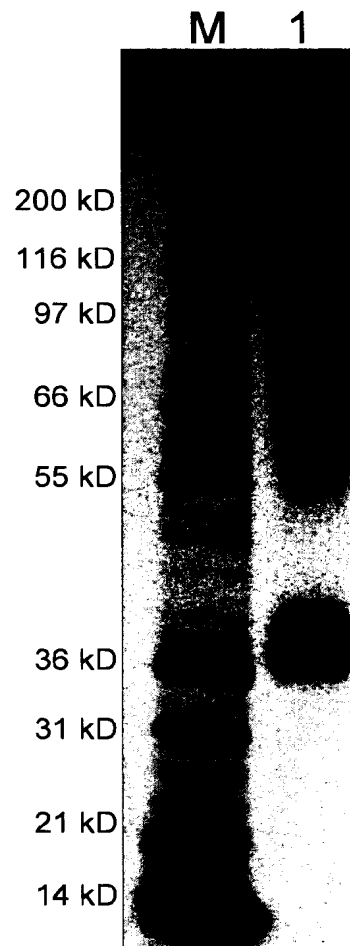
Figure 1
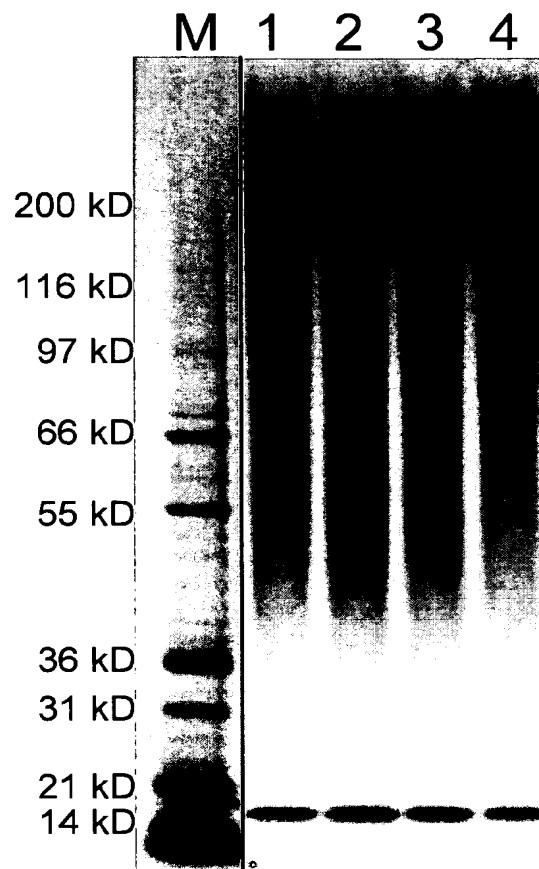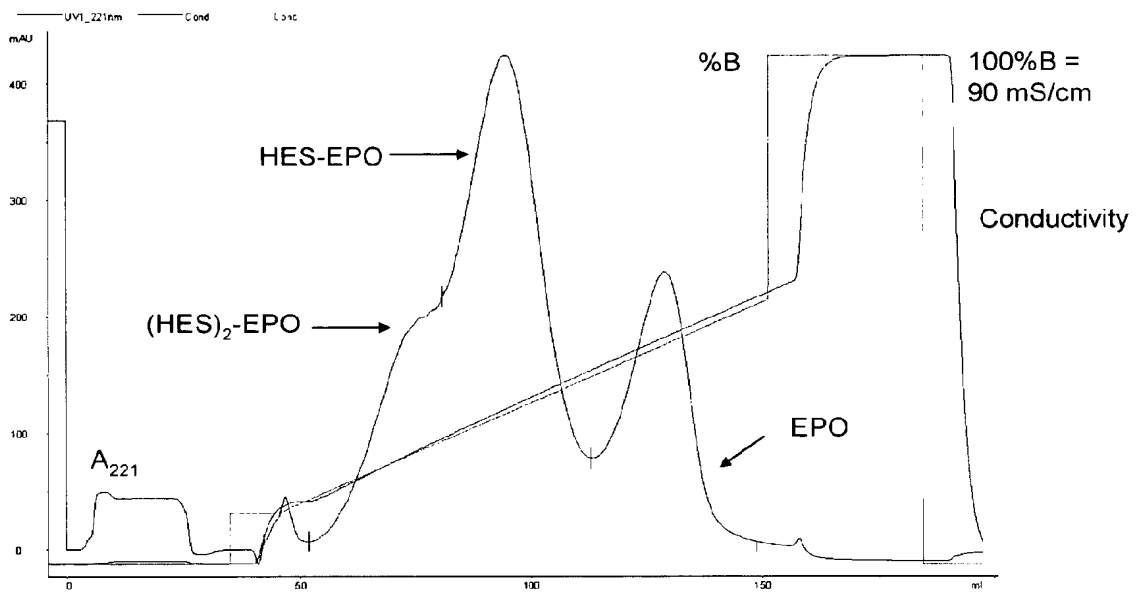
Figure 2

Figure 3

Figure 4

Figure 5

M   1

200 kD

116 kD
97 kD

66 kD

55 kD

36 kD

31 kD

21 kD
14 kD
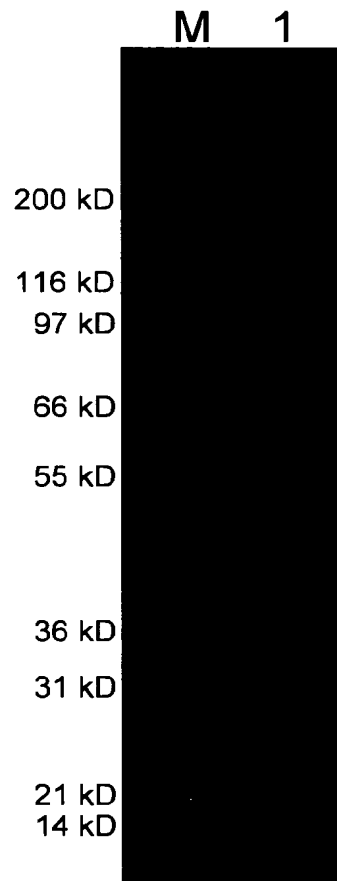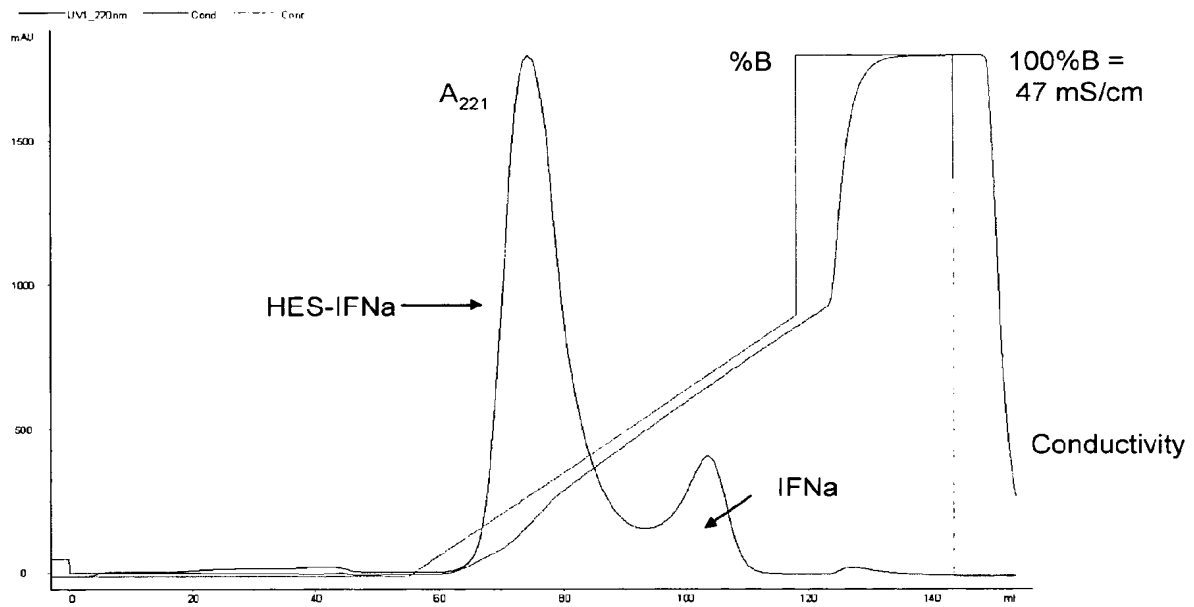
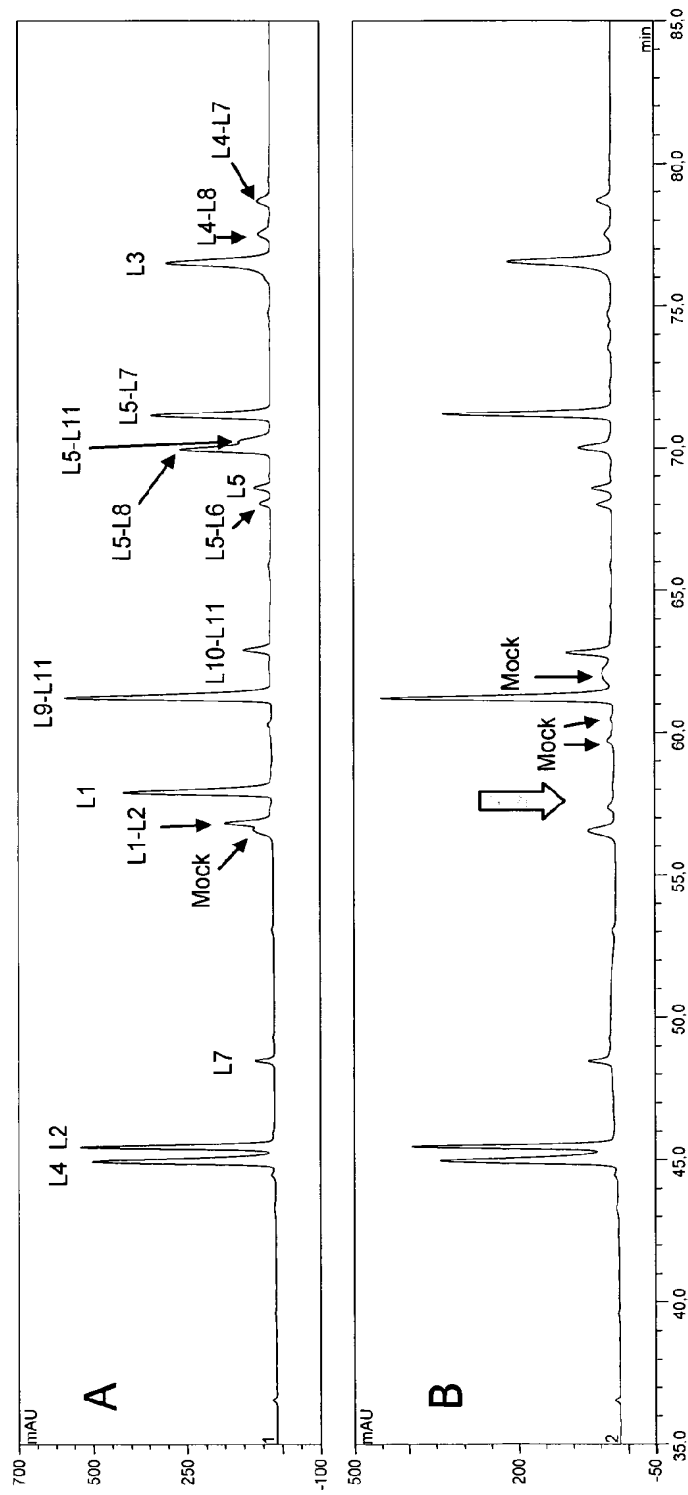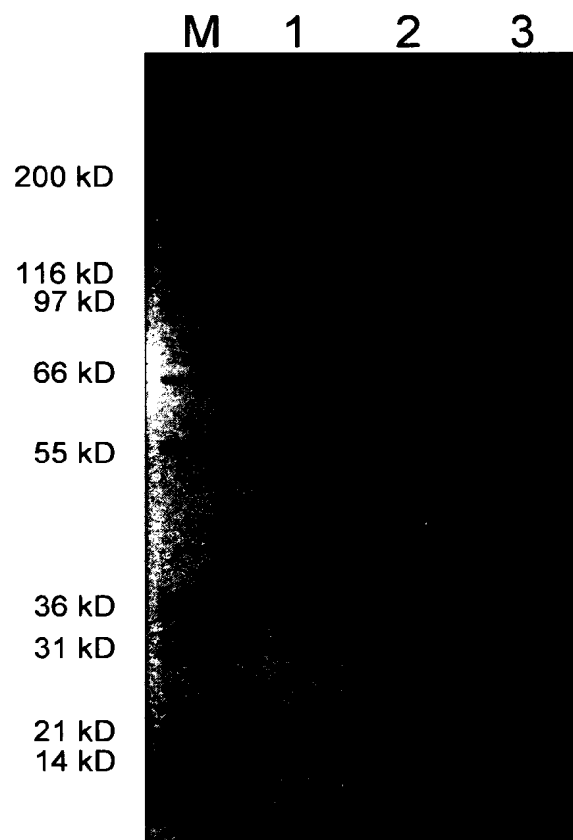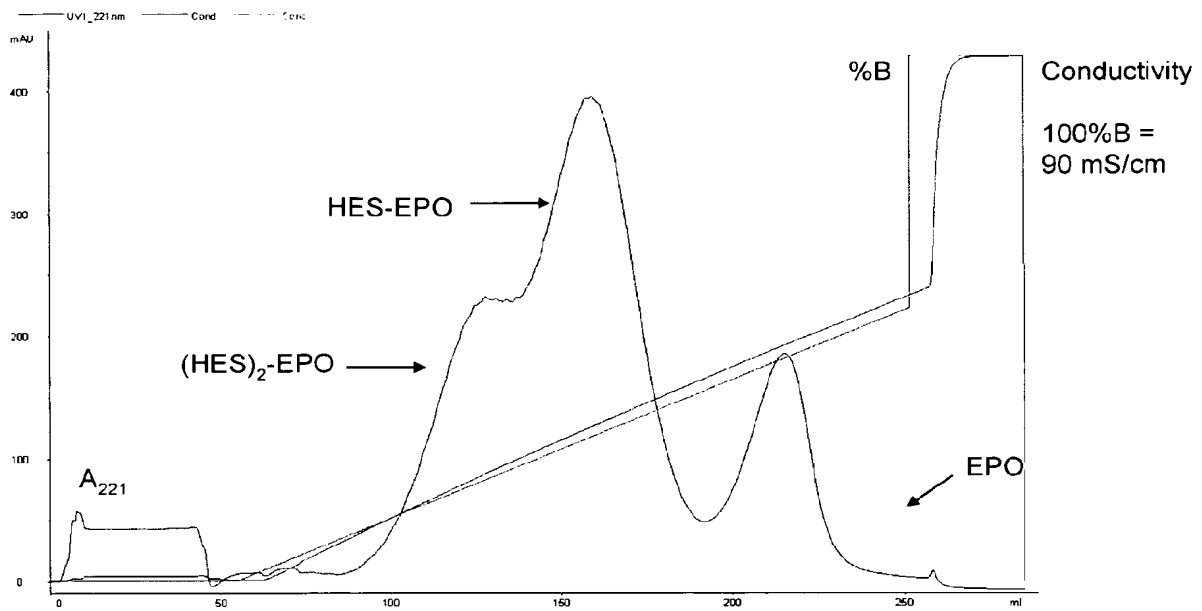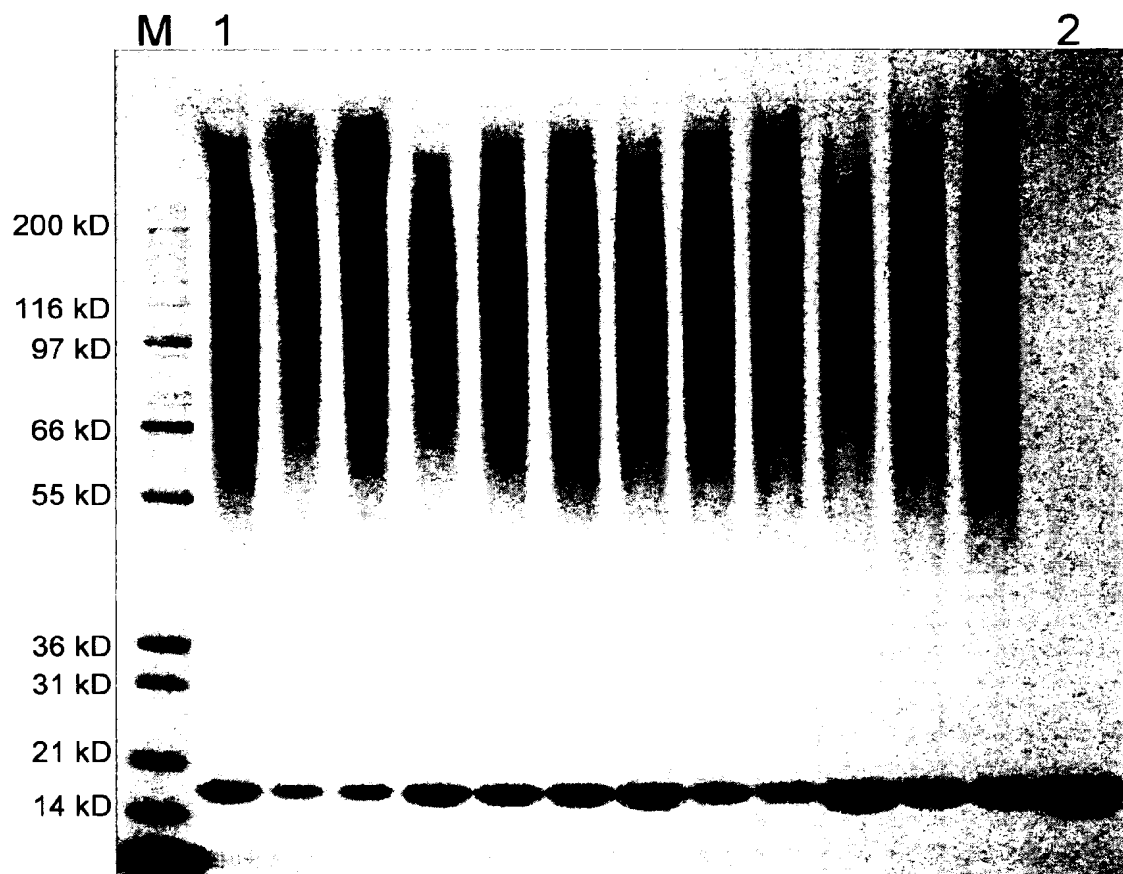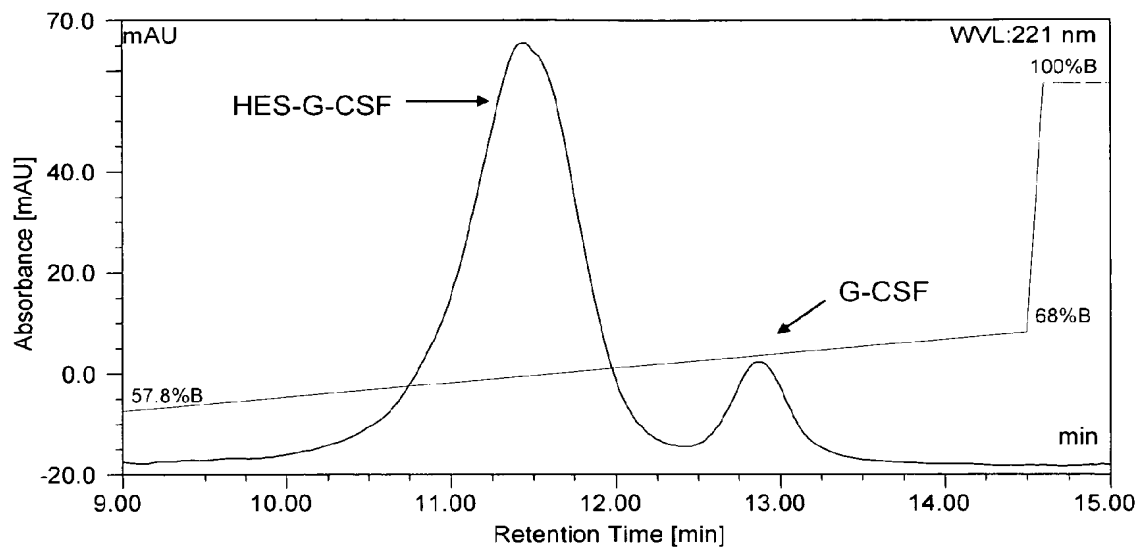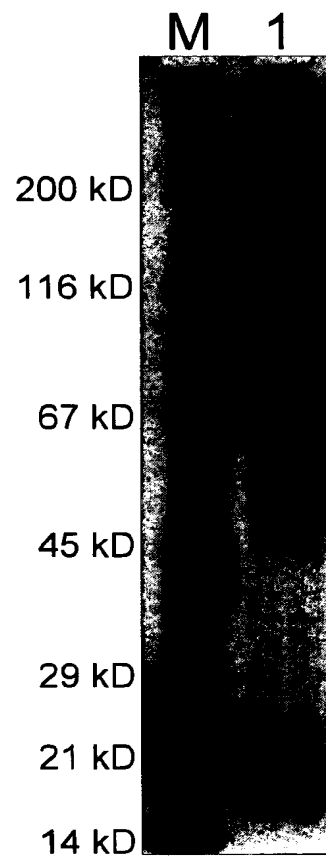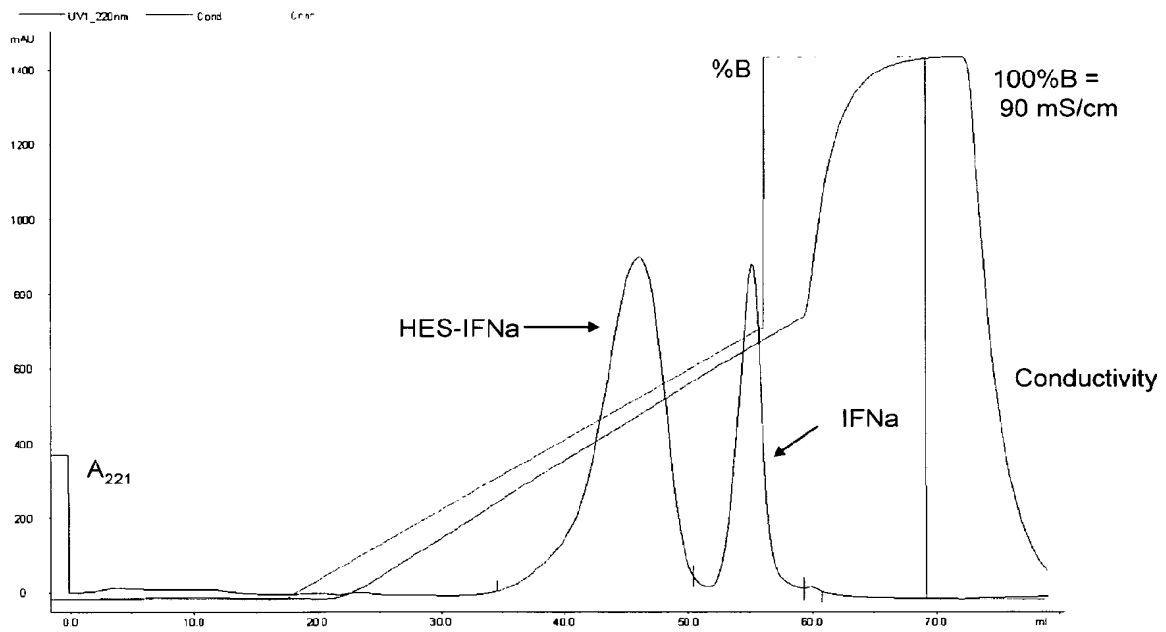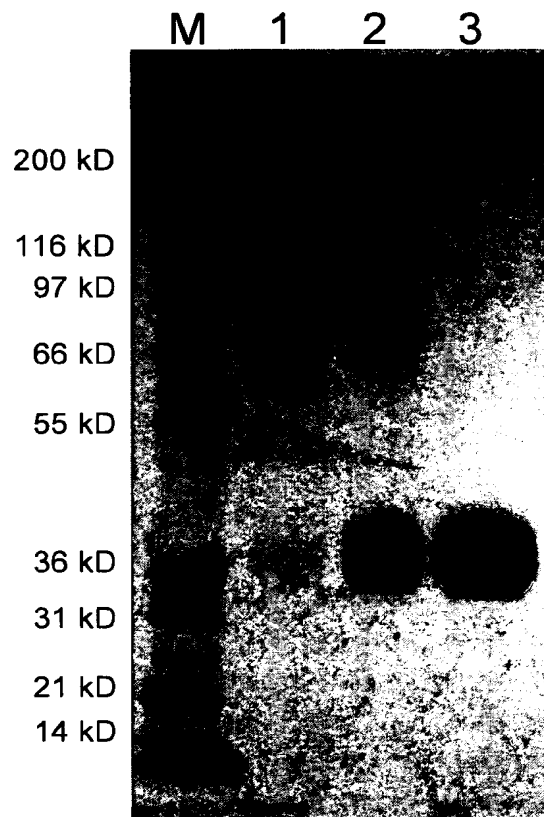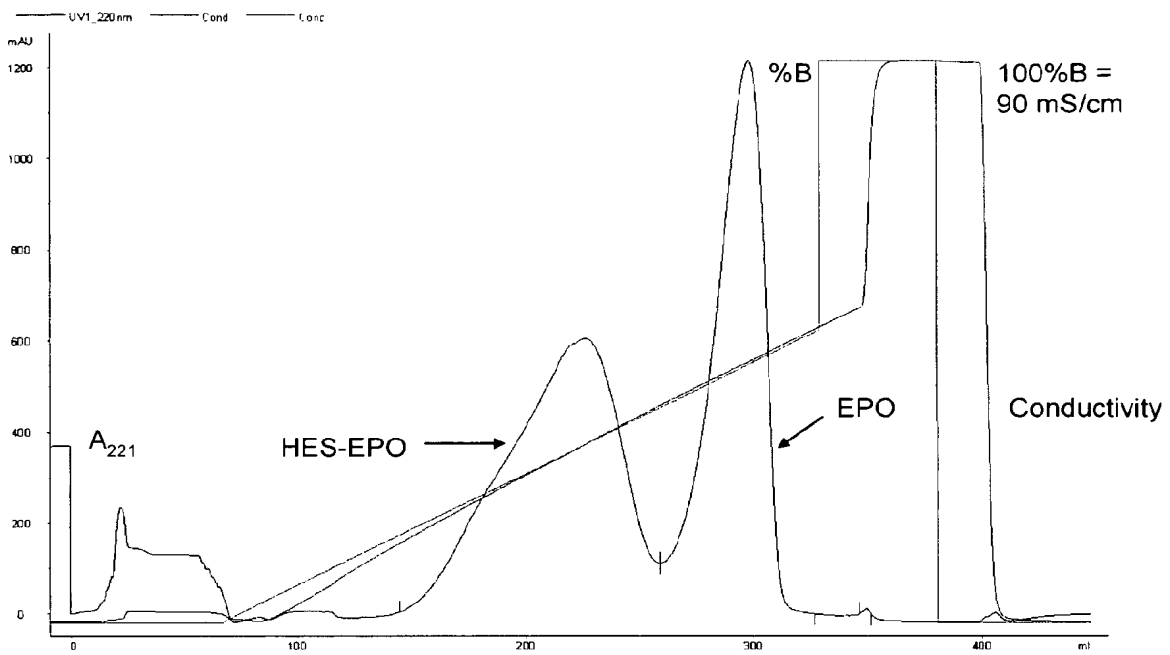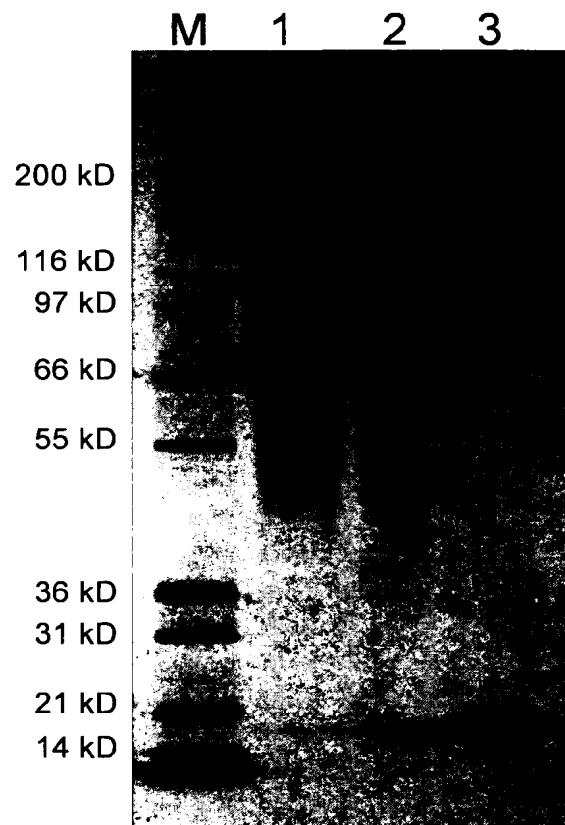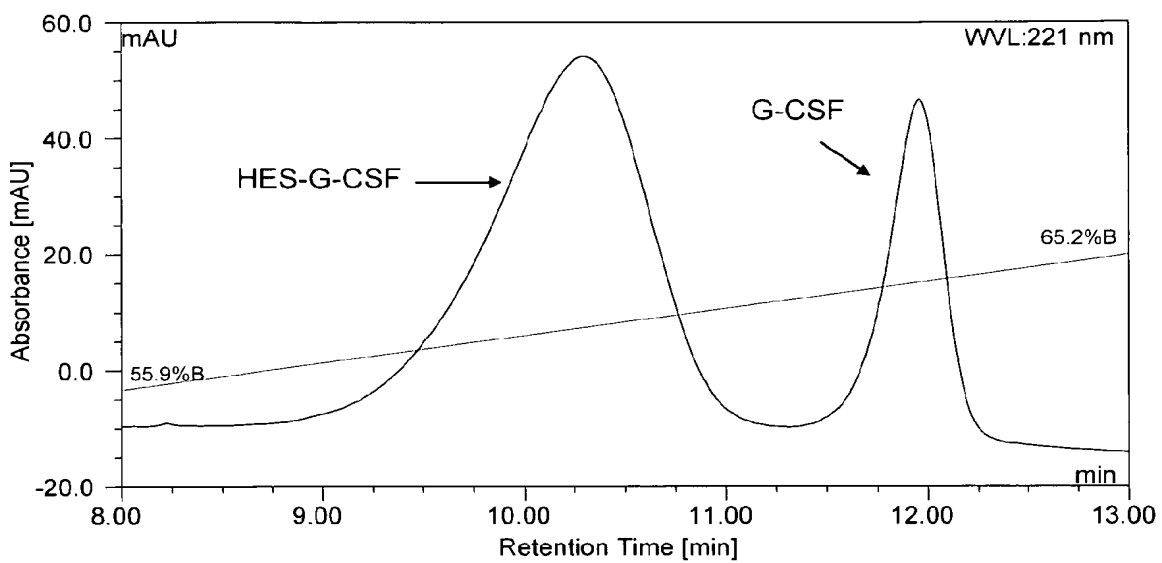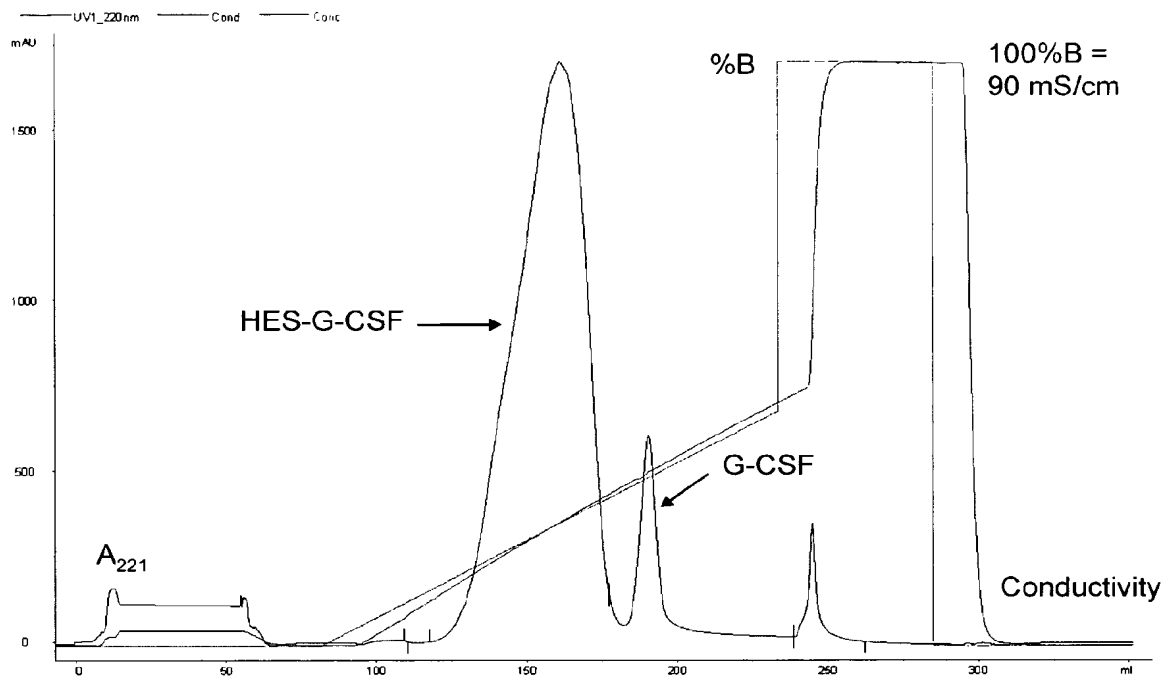**Figure 6**

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11
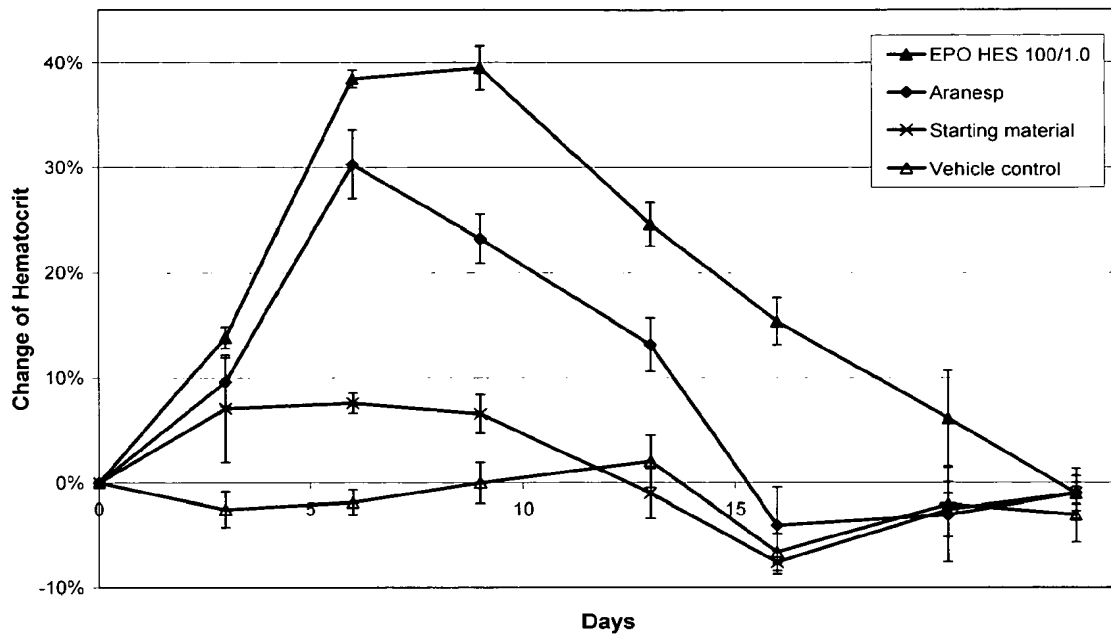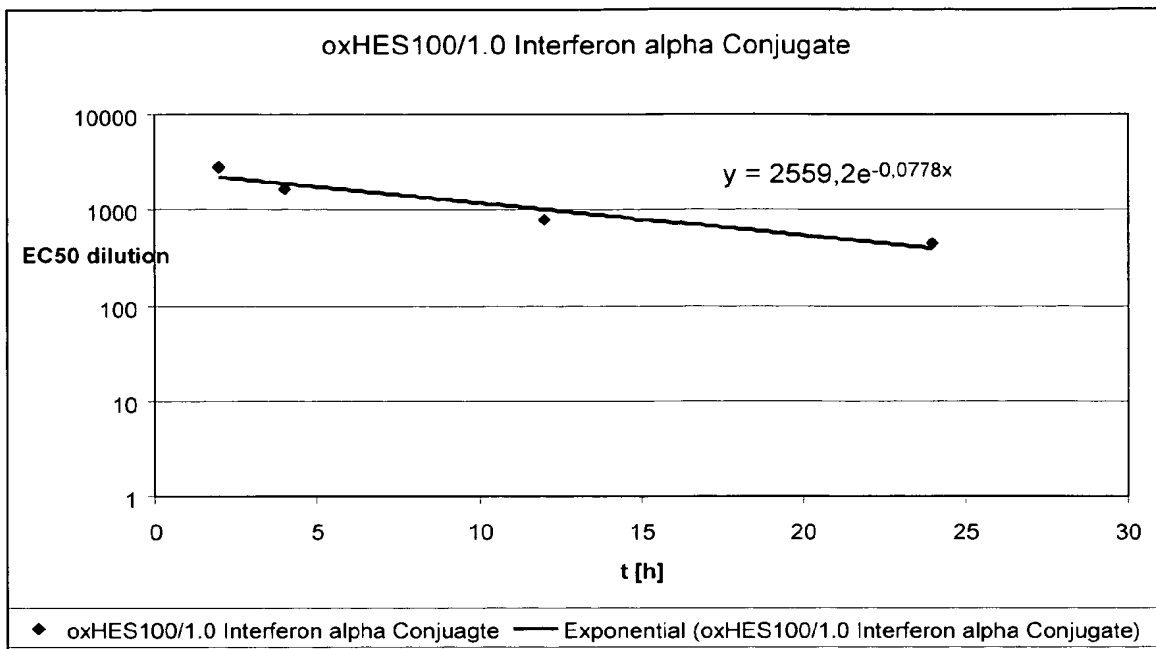
Figure 12

Figure 13

Figure 14

Figure 15

Figure 16

Figure 17

Figure 18

Figure 19

Figure 20

oxHES100/1.0 Interferon alpha Conjugate

$y = 2559,2e^{-0,0778x}$

◆ oxHES100/1.0 Interferon alpha Conjuagte ——— Exponential (oxHES100/1.0 Interferon alpha Conjugate)

Figure 21

in vitro activity oxHES100/1.0 Interferon-alpha Conjugate

Figure 22

HES100/1.0 Interferon alpha Conjugate

$y = 2123e^{-0,0716x}$

EC50 dilution

t [h]

◆ HES100/1.0 Interferon alpha Conjuagte ▬▬ Exponential (HES100/1.0 Interferon alpha Conjugate)

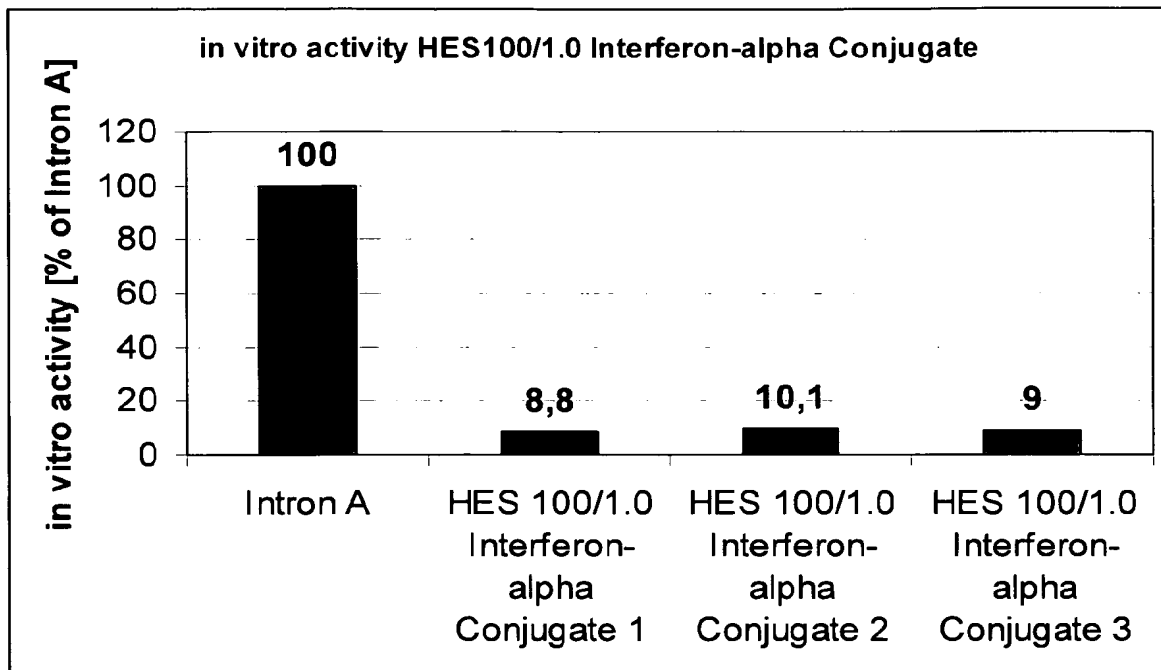Figure 23

in vitro activity HES100/1.0 Interferon-alpha Conjugate

Figure 24

Figure 25

Figure 26

Figure 27

EUROPEAN SEARCH REPORT

European Patent Office

Application Number

EP 07 02 4350
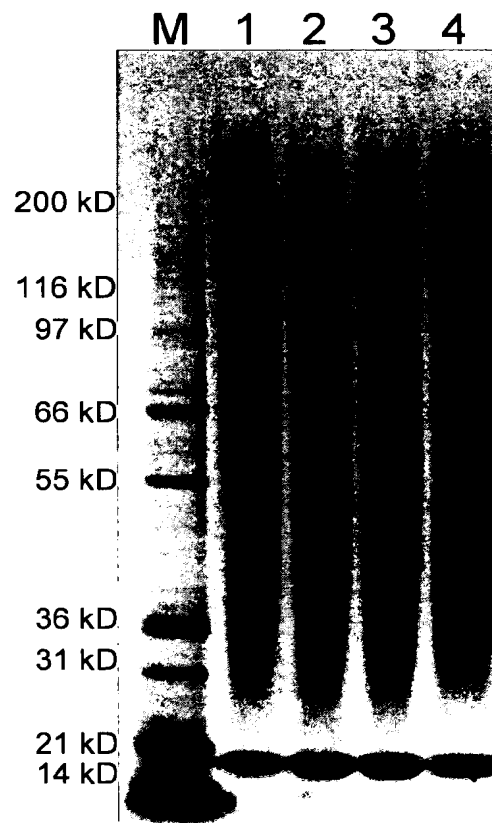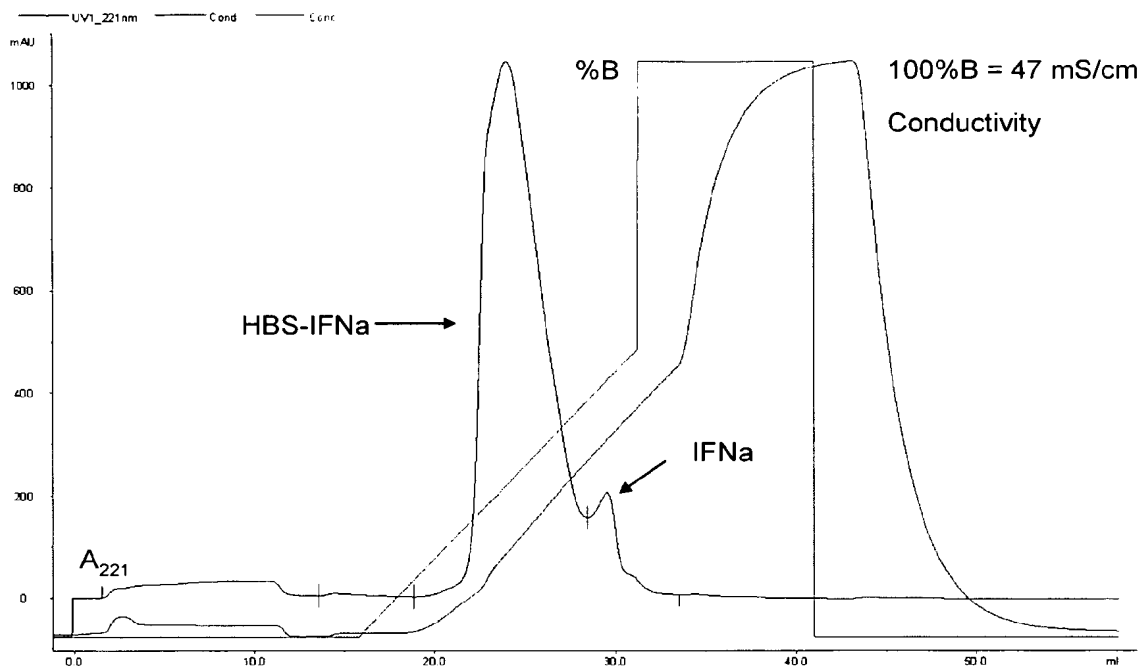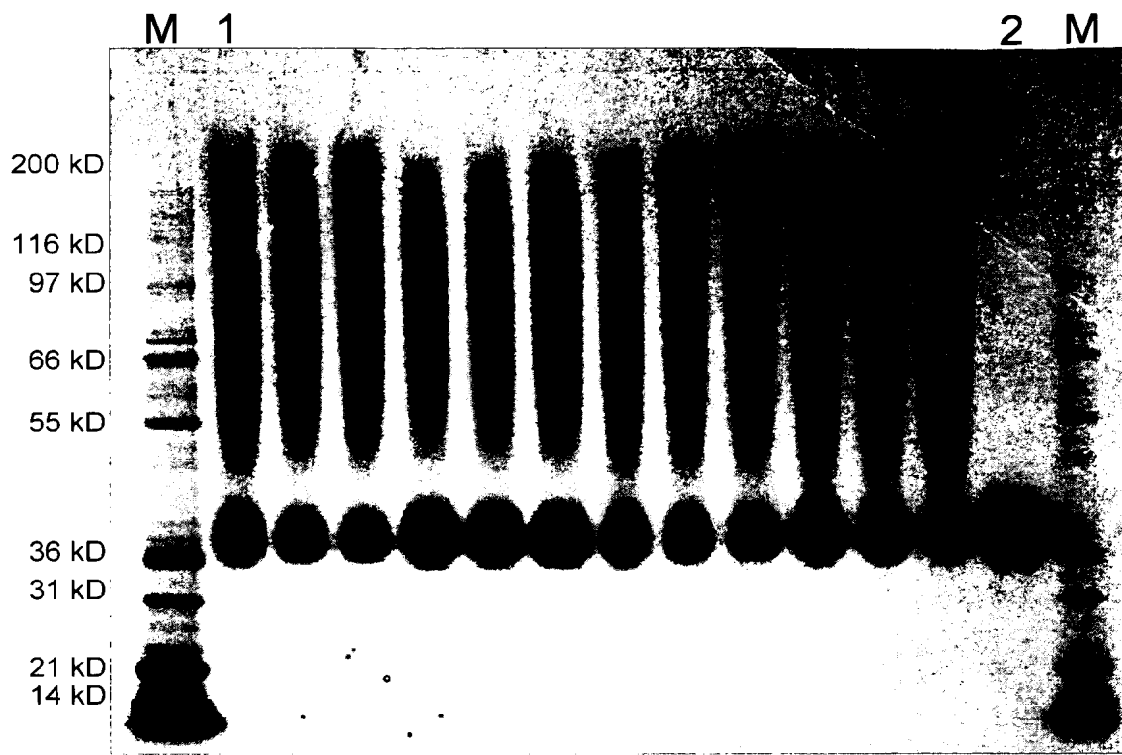
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D,X	US 2007/134197 A1 (EICHNER WOLFRAM [DE] ET AL) 14 June 2007 (2007-06-14)	29-37	INV. C08B31/12 A61K47/48
Y	* paragraphs [0003], [0006], [0008], [0010], [0013], [0385], [0389], [0410], [0422], [0500], [0502] * -----	1-28	
Y	US 2005/238723 A1 (ZANDER NORBERT [DE] ET AL) 27 October 2005 (2005-10-27) * paragraphs [0006] - [0009], [0011] - [0020], [0054], [0055], [0062], [0135], [0136], [0139] * -----	1-28	

TECHNICAL FIELDS SEARCHED (IPC)

C08B
A61K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	3 September 2008	Gerber, Myriam

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3

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EP 07 02 4350

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03-09-2008

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